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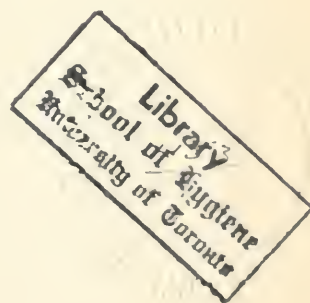
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IN CONJUNCTION WITH
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TABLE OF CONTENTS

NO. 1, JANUARY, 1917

PAGE

Comparison of methods for disinfecting swimming pools	
<i>Wallace A. Manheimer</i> - - - - -	1
Various methods of determining the trypanocidal activity of substances in vitro and their relationship to the chemotherapy of experimental trypanosomiasis	
<i>John A. Kolmer, Jay F. Schamberg, and George D. Raiziss</i> - -	10
Further observations on the adaptation of parasitic microorganisms to a lowered oxygen tension	
<i>William B. Wherry and Wade W. Oliver</i> - - - - -	28
The numeric relationship of infection to the chemotherapy of experimental trypanosomiasis	
<i>John A. Kolmer, Jay F. Schamberg, and George D. Raiziss</i> - -	35
Studies of the beta hemolytic streptococcus (Smith and Brown)	
<i>Wilson George Smilie</i> - - - - -	45
The bacteriology of pemphigus neonatorum	
<i>Frederick Howard Falls</i> - - - - -	86
Studies on immunity in typhus exanthematicus with reference to the antibodies in man and guinea-pig demonstrable by the Dale method	
<i>Bernard S. Denzer and Peter K. Olitsky</i> - - - - -	99
A small contact epidemic of typhoid fever	
<i>Mark F. Boyd</i> - - - - -	109
The etiology of acute epidemic poliomyelitis (Plates 1 to 4)	
<i>George Mathers</i> - - - - -	113

NO. 2, FEBRUARY, 1917

The virulence of diphtheria bacilli from diphtheria patients and diphtheria-carriers	
<i>George H. Weaver</i> - - - - -	125
The alleged detoxicating power of the thyroid gland	
<i>H. Raymond Basinger</i> - - - - -	131
An epidemic of acute diarrhea approaching in some cases a mild dysentery	
<i>Wilfrid Sadler, and R. F. Kelso</i> - - - - -	140
The passive transference of nonspecific antibodies	
<i>Peter K. Olitsky and Bernard S. Denzer</i> - - - - -	145
Comparative study of <i>Bacillus anthracis-symptomatici</i> and allied organisms with respect to gas-production	
<i>L. C. Todd</i> - - - - -	151
Transmission of pneumonic and septicemic plague among marmots	
<i>Frederick Ebersson and Wu Lien Teh</i> - - - - -	170
Plague poisons and virulence	
<i>Frederick Ebersson</i> - - - - -	180
The effect of various chemical substances on the hemolytic reaction	
<i>Noble P. Sherwood</i> - - - - -	185

	PAGE
Blood-culture studies on typhus exanthematicus in Serbia, Bulgaria, and Russia	
<i>George Baehr and Harry Plotz</i> - - - - -	201
Leukopenia and leukocytosis in rabbits	
<i>Clifford W. Wells</i> - - - - -	219

NO. 3, MARCH, 1917

Tuberculin hypersensitiveness as determined by intracutaneous tests of different degrees	
<i>Myer Solis-Cohen</i> - - - - -	233
Virulence and toxin-formation in <i>B. diphtheriae</i>	
<i>William E. Cary</i> - - - - -	244
Germicidal action and specific bacteriotropism of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchona derivatives on pneumococci in vitro. Studies in pneumonia, I	
<i>S. Solis-Cohen, John A. Kolmer, and George D. Heist</i> - - -	272
Various methods of determining the bactericidal action of substances in vitro and their relation to the chemotherapy of bacterial infections. Studies in pneumonia, II	
<i>John A. Kolmer, S. Solis-Cohen, and George D. Heist</i> - - -	293
The protective and curative value of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchona derivatives in experimental pneumococcus infections. Studies in pneumonia, III	
<i>S. Solis-Cohen, John A. Kolmer, and George D. Heist</i> - - -	313
The influence of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchona derivatives on leukocytosis and on phagocytosis and pneumococci in vitro. Studies in pneumonia, IV	
<i>John A. Kolmer, S. Solis-Cohen, and Edward Steinfield</i> - - -	333
Allergic skin reactions in pneumonia with type strains of pneumococci. Studies in pneumonia, V	
<i>Edward Steinfield, and John A. Kolmer</i> - - - - -	344

NO. 4, APRIL, 1917

The nonfiltrability of typhus virus	
<i>Peter K. Olitsky</i> - - - - -	349
The intracutaneous 'typhoidin' reaction	
I. The preparation and properties of the antigen	
II. The nature and specificity of the reaction	
III. The relation of cutaneous hypersensitiveness to experimental immunity and infection	
<i>K. F. Meyer and C. R. Christiansen</i> - - - - -	357, 391, 424
The treatment of experimental tuberculosis in guinea-pigs and rabbits with taurin, alone and in combination with gold chlorid and sodium oleate	
<i>Minokichi Takeoka</i> - - - - -	442
The differentiation of the paratyphoid-enteritidis group, I	
<i>Edwin O. Jordan</i> - - - - -	457

TABLE OF CONTENTS

vii

NO. 5, MAY, 1917

PAGE

The oxidase of <i>Rhus diversiloba</i> <i>James B. McNair</i> - - - - -	485
Anaphylatoxin and anaphylaxis. I. Trypanosome anaphylatoxin <i>F. G. Novy, P. H. DeKruif, and R. L. Novy</i> - - - - -	499
Anaphylatoxin and anaphylaxis. II. Agar anaphylatoxin: guinea-pig serum <i>F. G. Novy and P. H. DeKruif</i> - - - - -	536
Anaphylatoxin and anaphylaxis. III. Agar anaphylatoxin: rabbit serum <i>F. G. Novy and P. H. DeKruif</i> - - - - -	566
Anaphylatoxin and anaphylaxis. IV. Agar anaphylatoxin: rat serum <i>F. G. Novy and P. H. DeKruif</i> - - - - -	589
Anaphylatoxin and anaphylaxis. V. Effect of multiple doses of anaphylatoxin <i>F. G. Novy and P. H. DeKruif</i> - - - - -	618
Anaphylatoxin and anaphylaxis. VI. Effect of intravenous injections of agar <i>F. G. Novy and P. H. DeKruif</i> - - - - -	629

NO. 6, JUNE, 1917

Anaphylatoxin and anaphylaxis. VII. Peptone anaphylatoxin <i>F. G. Novy, P. H. DeKruif, and F. O. Novy</i> - - - - -	657
Anaphylatoxin and anaphylaxis. VIII. The primary toxicity of normal serum <i>P. H. DeKruif</i> - - - - -	717
Anaphylatoxin and anaphylaxis. IX. Specific anaphylactic shock <i>F. G. Novy and P. H. DeKruif</i> - - - - -	776
Anaphylatoxin and anaphylaxis. X. Anaphylatoxin and amino-nitrogen <i>P. H. DeKruif and W. M. German</i> - - - - -	833

COMPARISON OF METHODS FOR DISINFECTING SWIMMING POOLS*

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From the Research Laboratory of the Department of Health of New York City

ULTRA VIOLET LIGHT

Ultra violet light has been used abroad for the disinfection of drinking water and in Paris for the disinfection of a swimming pool. In this country, where it has recently been introduced, its application to the disinfection of swimming pools is the most modern device adopted for that purpose. In the matter of operation and handling, ultra violet light would be the most satisfactory method of disinfection thus far introduced. In the first place, nothing which could arouse objection from bathers is added to the water, and in the second place it is automatic—neither too much nor too little could be added by a possibly bungling attendant, since after turning on the light, he would need to give no further attention to the apparatus. Accordingly, the determination of its cost of operation, bacterial efficiency, etc., have been investigated with the utmost interest.

Tests at the 57th Street Y. M. C. A.—The capacity of this pool is approximately 75,000 gallons. The water is circulated through the filters every 24 hours and is thereby maintained clear. It is drawn from the bottom of the deep end and returned after filtration to the surface of the shallow end, being there discharged from a pipe about 2 feet above the surface of the pool, the latter measure insuring considerable aeration of the water. At the time of these tests the water had been in the pool for about 6 months.

Samples of water were collected from the pool at the deep end, from a tap on the pipe returning from the filter, and from the pipe discharging into the pool after the water had been exposed to the ultra violet light. From the analyses of these samples it was possible to determine the number of bacteria in the pool, the amount of reduction due to refiltration, and the amount of reduction, if any, due to exposure to the ultra violet light. Similar analyses were made in all tests on ultra violet light.

Series 1. A count of 20 bacteria to the cubic centimeter in water from the pool at 37 C., was reduced by filtration to 5, and by exposure to ultra violet light, to 2. This reduction of 3 bacteria to the cubic centimeter as a result of exposure to the light, in plate counts might readily be attributed to normal experimental error in pouring plates.

Series 2. Here a count of 250 bacteria to the cubic centimeter for the pool sample at incubator temperature fell to 40 during filtration, and rose

* Received for publication September 20, 1916.

TABLE 1

BACTERIAL COUNTS IN Y. M. C. A. POOL AFTER INSTALLATION OF VIOLET LIGHT APPARATUS *

Source of Sample Tested	1st Series		2nd Series			3rd Series		
	37 C.	Colon Bacilli per c.c.	37 C.	20 C.	Colon Bacilli per c.c.	37 C.	20 C.	Colon Bacilli per c.c.
From pool.....	20	* 0	250	231	0	150†	416†	½
From filter.....	5	0	40	32	0	5	52	0
From violet light.	2	½	42	53	1	15	70	0

* Water in pool 6 months. † Pool had been agitated by scooping up dirt from bottom.

BACTERIAL COUNTS IN THE SAME POOL BEFORE USE OF ULTRA VIOLET LIGHT FOR DISINFECTION ‡

Day of Test	1st	2nd	3rd	4th	5th	6th	7th
At 37 C.	30	700	4000	2500	2600	5000	14000
At 20 C.	300	6000	44000	150000	150000	170000	31000

‡ Pool emptied every 2 weeks.

slightly, to 42, after exposure to the ray. The room-temperature counts showed about the same result—231 bacteria to the cubic centimeter in the pool sample, 32 in the sample from the filter, and 53 in the sample after exposure to the lights.

Series 3. In this series the results were approximately the same as before. The water in the pool, which had been agitated by scooping dirt from the bottom, contained 150 bacteria to the cubic centimeter at 37 C. and 416 at 20 C. After filtration the counts were 5 and 52, respectively, and after exposure to the ray 15 and 70. Colon bacilli were irregularly present in the 1-c.c. samples.

Tests at the New York Athletic Club.—In order more thoroughly to investigate the value of the machine for disinfection of water, additional series of tests were planned at the New York Athletic Club.

Series 1. Water from the pool gave a count at 37 C. of 345 bacteria to the cubic centimeter, and at 20 C. of 880 bacteria to the cubic centimeter. After refiltration the counts were 250 and 500. After exposure to the ray the counts were 240 and 730.

Series 2. The experiment was repeated as a control before adding a large number of colon bacilli to the water. Again there was no noticeable reduction in the bacterial counts after exposure to the ray.

Series 3. In order to produce test conditions under which results would be more striking, a large number of colon bacilli from a stock culture grown on agar were added to the pool. The data obtained indicate that while the filters were chiefly responsible for the reduction, the ultra violet light did play some part in lowering the counts.

The shortness of the time of exposure of the water to the light probably accounts for the rather poor results. That the machine is of some value in purifying the water is evident from a comparison of the bacterial counts taken at the 57th street Y. M. C. A. before the apparatus had been installed with those after the machine was in use (Table 1). The difference between the two groups is striking. Since

TABLE 2

BACTERIAL COUNTS IN N. Y. A. C. POOL AFTER INSTALLATION OF ULTRA VIOLET LIGHT APPARATUS

Source of Sample Tested	1st Series			2nd Series			3rd Series*		
	37 C.	20 C.	Colon Bacilli per c.c.	37 C.	20 C.	Colon Bacilli per c.c.	37 C.	20 C.	Colon Bacilli per c.c.
From pool.....	345	880	0	600	620	0	3750	2400	1000
From filter.....	250	500	0	240	470	0	240	470	10
From violet light	240	730	0	200	450	1	200	450	1

* Colon bacilli added to the pool.

BACTERIAL COUNTS IN THE SAME POOL BEFORE THE USE OF ULTRA VIOLET LIGHT FOR DISINFECTION †

Day of Test	1st	2nd	3rd	4th	5th	6th	7th	8th
At 37 C.	150	1000	200	180	200	300	180	500
At 20 C.	350	10000	6000	1000	230	3000	300	2000

† Pool continuously diluted with warm filtered water, and emptied every 2 weeks.

the use of ultra violet light was the only change in technic, it would appear that this light exerts a steady reducing effect on bacterial pollution.

In the test in the New York Athletic Club the results would point the other way (Table 2). Here, however, the pool when not treated with ultra violet light was continuously diluted with fresh warm water, so that the comparison of bacterial counts before and after, in this instance, is not easily made.

In view of the facts thus far advanced it would seem desirable to increase the time of exposure of the water to the light.

"LECTROCIDE"

"Lectrocide" is a chemical manufactured through electrolysis of brine water (sea water). It is a greenish chemical with a chlorin odor, altho its main ingredient is sodium hypochlorite.

Two methods of applying this chemical to the swimming pool were tried: (1) The chemical was introduced into the bottom of the deep end through a long pipe. Water from the pool was then poured through so as to drive the chemical below the surface of the pool. Tests lasting for 1 week were conducted while this method of treating the water was in use. (2) During the following week the chemical was introduced into the water through an automatic feed pot attached to the pipe leading from the filter to the pool. The valve on the feed pot was arranged so that the chemical was completely discharged into the water in 24 hours.

The data collected (see coefficient in Table 3) during 15 days of test indicate that the introduction of this chemical into the water in single daily doses is superior to the slow continuous adding of the chemical from a feed pot.

The lectroicide was added so that there was 1 part of chlorin to 1,000,000 parts of water. The bacterial contamination in the swimming pool when treated with lectroicide remained low throughout the whole series of experiments (Table 2, Nos. 1, 2, and 3).

COPPER SULFATE

Experiments were conducted with copper sulfate similar to those conducted with lectroicide and similar to those conducted with chlorin gas and calcium hypochlorite, to be described later. The object was to secure data upon which comparison of relative efficiencies could be based.

Copper sulfate in concentration of 1 part in 100,000 parts of water by weight was added to the pool daily. It was dissolved in water and added in the same ways as lectroicide; that is, as a single daily dose for the first week and from the mixing pot as a continuous gradual dose during the second week. In direct contrast to the experiment with lectroicide, here the continuous slow adding of chemical was more effective than single daily dosing. Evidently the efficiency of chemical disinfection is dependent not only on the chemical employed, but on the method of adding it to the water.

The efficiency of copper sulfate itself, however, is low when 1 part in 100,000 is used. Bacterial pollution was higher throughout the 2 weeks of test than it was when sodium hypochlorite was substituted. Furthermore, the cost per day for treatment with copper sulfate was \$1.04—nearly 8 times that for treatment with “lectroicide.” The water was yellowish and its transparency was reduced to such an extent as to draw complaints from the patrons. When the pool was emptied, the tiles were stained, so that scrubbing with oxalic acid was necessary in addition to the ordinary labor of cleaning the pool. In short, as will be seen later, copper sulfate showed the poorest efficiency and the highest cost of application of all chemicals used in pool-disinfection. (See coefficient values in Table 3. The results with chlorin gas, Table 2, No. 6, should not be included in the comparison because of defective apparatus.)

TABLE 3

COMPARISON OF THE RESULTS OF VARIOUS METHODS OF DISINFECTING SWIMMING POOLS

Day	1. "Lectrocide" Added in Single Daily Doses		2. "Lectrocide" Added in Single Daily Doses		3. "Lectrocide" Added From Feed Pot		4. Copper Sulfate in Single Daily Doses	
	37 C.	20 C.	37 C.	20 C.	37 C.	20 C.	37 C.	20 C.
1st.....	350	900	2500	660	18000	16500	2000	28000
2nd.....	10000	8000	5200	8000	5000	13200	40000	84000
3rd.....	10000	7000	2400	2700	400	1400	30000	70000
4th.....	7500	400	1800	2400	80	2500	30000	42000
5th.....	200	1000	660	2200	29000	89000	56000	66000
Av. daily attendance	498		674		458		500	
Capacity....	52000		52000		52000		52000	
Coefficient...	470		22		1700		4700	

Day	5. Copper Sulfate From Feed Pot		6. Anhydrous Chlorin From Needle Valve		7. Calcium Hypo- chlorite		8. Calcium Hypo- chlorite	
	37 C.	20 C.	37 C.	20 C.	37 C.	20 C.	37 C.	20 C.
1st.....	750	4000	20	400	1000	200	1400	9200
2nd.....	6500	28000	16400	10000	2400	1600	2200	3400
3rd.....	26000	47000	202000	400000	200	1400	300	900
4th.....	8000	20000	160	400	3500	3600	6000	18000
5th.....	15000	28000	4800	36000	80	6000	20	5200
Av. daily attendance	514		1261		493		337	
Capacity....	52000		55000		80000		75000	
Coefficient...	2000		2900		360		86	

Day	9. Calcium Hypochlorite		10. Calcium Hypochlorite		11. Calcium Hypochlorite	
	37 C.	20 C.	37 C.	20 C.	37 C.	20 C.
1st.....	40	60	250	3600	200	3800
2nd.....	500	1400	1500	1100	150	1400
3rd.....	2	70	20	200	2	180
4th.....	70	240	40	50	10	10
5th.....	100	1500	10	1200	10	9000
Average daily attendance.....	338		200		130	
Capacity.....	75000		40000		40000	
Coefficient.....	86		160		450	

Note:—Since the bacterial pollution in a pool varies with the attendance and with the volume of the water in the pool, the coefficient was computed according to the following formula:

$$\text{Coefficient} = \frac{\text{Average daily bacterial count}}{\text{Average daily attendance}} \times \frac{\text{Volume of pool}}{1000}$$

The coefficients are merely rough estimates, but they furnish a simple basis of comparison in determining the efficiency of the different methods of treating the pool.

CHLORIN GAS

Chlorin gas was injected into tap water which was under sufficient pressure to feed into the pipe leading from the filtering plant to the pool.

The analyses conducted for 1 week on this pool were unsatisfactory because of defective apparatus. According to the engineer in charge of baths, the quality of anhydrous chlorin has deteriorated since the war, frequently containing a heavy oil which is carried over when the chlorin tanks are filled. This oil clogs the needle valve of the injector causing interruption in the flow of the gas. For this reason, the data collected do not indicate the efficiency of chlorin gas for pool-disinfection. In tests on another pool,¹ however, chlorin gas proved to be an efficient and easily handled disinfectant.

OZONE

Series of tests on ozone applied to drinking water were conducted. This chemical has not yet been applied to the disinfection of swimming pools, but its use would have so many obvious advantages that I have undertaken to experiment with it. While there are no figures as yet on cost of operation or experience of its reliability and ease of control as applied to swimming pools, the tests thus far conducted indicate a very high efficiency on the part of ozone as a purifier of water. Within a short time, I hope to report more extensively on this subject.

DISCUSSION OF COMPARATIVE EFFICIENCY AND COMPARATIVE COSTS OF THE VARIOUS METHODS

Copper Sulfate.—Recently Thomas² and Levine,³ contrary to most observers, concluded that copper sulfate is superior to calcium hypochlorite for the disinfection of swimming pools. Levine reports satisfactory results at Iowa State College, using 1 part of CuSO_4 to 1,000,000 parts of water.

The results set forth in this paper show that even when 1 part of CuSO_4 to 100,000 of water was used, the bacterial pollution in the pool exceeded that with all other methods of disinfection.

DeWitt and Sherman⁴ showed that while very small dilutions of some chemicals destroyed tubercle bacilli, 5% solution of copper sulfate

¹ Jour. Infect. Dis., 1914, 15, p. 159.

² Jour. Ind. and Eng. Chem., 1915, 7, p. 476.

³ Jour. Infect. Dis., 1916, 18, p. 293.

⁴ Ibid., 1914, 15, p. 245; 1916, 18, p. 368.

was necessary, even when the time of exposure was 24 hours. In their more recent paper on the subject they conclude: "In the short time of an ordinary laboratory experiment and with the small amount of material usually employed, copper is unreliable and unsatisfactory both as a bactericide and as a fungicide. One percent fails to kill all organisms within 15 minutes."

Johnson and Copeland got unsatisfactory results with sewage effluent even when using 1 part CuSO_4 to 25,000 parts of the effluent.

Clark and Gage,⁶ after an elaborate study of the effect of copper sulfate in various dilutions on water from various sources, conclude as follows: "In conclusion, the writers believe that the treatment of water with copper sulphate . . . has little practical value for the following reasons:

"I. The use of any method of sterilization which is not absolutely effective is dangerous in the hands of the general user, tending to induce a feeling of false security and leading to the neglect of ordinary precautions which would otherwise be employed.

"II. Although the removal of *B. coli* and *B. typhosus* is occasionally accomplished by dilute solutions of copper sulphate, these organisms may both live for many weeks in water containing copper sulphate in greater dilutions than 1:100,000; and in order to be safe dilutions of 1:1000 must be used, in which case the water becomes repugnant to the user because of its strongly astringent taste.

"III. In some instances very dilute solutions of copper sulphate. [e. g., 1:1,000,000 as used by Levine for treating a swimming pool] . . . appear to have a decidedly invigorating effect on bacterial activity, causing rapid multiplication, when the reverse would have been true had the water been allowed to stand the same length of time without any treatment."

The conclusion of Clark and Gage, that high dilutions of copper sulfate are valueless for disinfecting water, agrees with the results which I obtained in the tests on the 23rd Street Baths. Furthermore, as previously stated, copper sulfate costs about \$1.04 a day for a 50,000-gallon pool (before the war about 50 cents) when 1 part of CuSO_4 is added daily to 100,000 parts of water. The water takes on a yellowish appearance and becomes less transparent. The tiles lining the pool are somewhat discolored.

⁵ Ibid., 1905, Suppl. 1, p. 327.

⁶ Ibid., 1906, Suppl. 2, p. 175.

Anhydrous Chlorin.*—This is the cheapest of the chemicals to use, costing about 0.5 of a cent per day for a 50,000-gallon pool. When it is free from oil, it is easily controlled and very satisfactory to operate. As stated, stoppage of the needle valve and consequent interruption of the flow of gas make the determination of its efficiency impossible in these tests. In tests on another pool, however, it proved highly satisfactory.¹

*"Lectroside."**—This costs now 18 cents a day (before the war about 9 cents) for a 50,000-gallon pool. It is easily handled, causes no objectionable odor, and has a high coefficient of disinfection for swimming-pool water.

Chlorid of Lime, Calcium Hypochlorite.*—This is a very cheap disinfectant and one showing by comparative test a high grade of efficiency. It costs about 8 mills per day for a 50,000-gallon pool. It is more difficult to handle than any of the foregoing chemicals because of its unpleasant odor when improperly added to the pool and because of the stoppage of the filters if the lime sludge is thrown into the filtering circuit. These objections can be easily overcome if the following procedure is adhered to:

Mix the chemical with water thoroughly in a large pail, stirring with a stick so that the active material is brought into solution. Do this in the open air, or in some place remote from the room containing the pool, since, when the chemical is mixed in the room, chlorin gas escapes into the air, causing objection on the part of the bathers to the chemical disinfection.

After the chemical has been thoroughly mixed with water, filter it through cheese cloth into another container, so that the lime sludge can be discarded.

Introduce the chemical into the water at the deep end of the pool through a large pipe extending nearly to the bottom of the pool. A funnel may be needed to pour the chemical in. Do not withdraw the pipe until after pouring some fresh water through it. This method obviates the necessity of pouring the chemical on the surface of the water. The presence of the chemical, either in solution or as a powder on the surface of the water, causes unpleasant odors in the air. Another and simpler method is to drop the pail containing the chemical forcefully into the water. In this way very little of the chemical remains on the surface. With this method and care to prevent odors of the chlorid of lime from escaping into the air, more than a dozen sanctioned Mikveh⁷ baths in New York City were treated without complaint from the patrons.

CONCLUSION

The value of ultra violet light as a disinfectant in swimming pools has not yet been definitely determined. (a) Swimming pools

* Chlorine gas and the hypochlorites were added to the water so that there was 1 part of available chlorin to 1,000,000 parts of water.

⁷ Survey, 1914, 34, p. 482.

equipped with ultra-violet-light apparatus showed lower bacterial pollution during its use, than before its use. (b) A somewhat longer exposure of the water to the light would seem desirable; in most instances bacterial reduction was not observed after the water had passed through the ultra-violet-light apparatus.

In regard to the method of adding chemicals to the water, it appears that the value of the slow continuous addition or of the single daily dosing varies with the chemical used. Copper sulfate gave the better results when added gradually and continuously, while sodium hypochlorite gave the better results with single daily dosing.

The relative efficiency of chemicals for water disinfection may be expressed as follows:

(a) Calcium hypochlorite,—high efficiency, low cost, not much care necessary in handling.

(b) Chlorin gas,—efficiency high, cost very low, easily handled.

(c) "Lectrocid",—cost 10 times as much as for hypochlorite, high efficiency, very easily handled.

(d) Copper sulfate,—cost high, efficiency low, stains tiles, causes reduction in transparency of water, easily handled.

Final decision on a standard method for pool disinfection has not yet been reached, and can not be until after ozone and other methods still under investigation, have been fully tested. In the 9 pools examined, refiltration was practiced in all cases, a procedure which should be standard in all indoor pools.

VARIOUS METHODS FOR DETERMINING THE TRY-
PANOCIDAL ACTIVITY OF SUBSTANCES IN
VITRO AND THEIR RELATION TO THE
CHEMOTHERAPY OF EXPERIMENTAL
TRYPANOSOMIASIS *

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During the course of chemotherapeutic studies, compounds possessing a well-defined parasitropic effect may be prepared which prove so highly organotropic or toxic for the test animal that the minute doses tolerated fail to exert an appreciable influence on the parasites. The mercurials, which are known to exert a powerful bactericidal effect in vitro, are substances of this class, being so highly toxic that doses greater than 0.0004 gm. per 100 gm. of white rat are apt to prove fatal and yet are too small materially to influence the course of experimental trypanosomiasis.

In order, therefore, to avoid the mistake of discarding new compounds possessing a high degree of trypanocidal activity because they are highly toxic for experimental animals, a study of various methods of determining the trypanocidal activity of a drug independent to some extent of its toxicity for the body cells of the host, was undertaken. In the event of the preparation of a new compound that is possessed of a high degree of parasitropism, but is also highly toxic, the efforts of the experimental chemotherapeutist are devoted to lowering the toxicity to a sufficient extent to permit of the administration of the drug to living animals.

With the purpose, then, of devising a technic for determining the parasitocidal activity of a chemical independent of its toxicity for body cells, we have studied various methods of determining the trypanocidal activity of substances in vitro. There is considerable evidence at hand that substances exhibiting a marked parasitocidal effect in vitro are likely to act in a similar manner in vivo, provided the toxicity is sufficiently low to permit of the administration of fair-sized doses; this is true to a certain extent of arsenobenzol, as will be shown

* Received for publication October 4, 1916.

later in this communication, and has been found true of ethylhydrocuprein hydrochlorid and other quinin derivatives in the treatment of experimental pneumococcus infections.¹

Since the cultivation of pathogenic trypanosomes, as *T. equiperdum* and *T. brucei*, on artificial media yields irregular results, our methods were designed to determine the influence of a drug upon trypanosomes in vitro by intraperitoneal injection of drug and trypanosome into white rats—such influence to be observed in loss of motility and structural changes, determined by direct microscopic examination, and more particularly in whether or not the trypanosomes were killed.

Several of these methods are similar to those devised by one of us (Kolmer²) for determining the bactericidal action of substances in vitro with reference to the chemotherapy of bacterial infections. The microscopic method described herein is essentially similar to that described by Kolmer and Smith³ in a study of the trypanocidal activity of emetin in vitro.

MICROSCOPIC METHOD

Technic.—A series of dilutions of the chemical in 0.85% sodium-chlorid or Ringer's solution and twice as strong as those desired for action on the trypanosomes, is prepared in amounts of 0.5 c.c. and placed in small sterile test tubes in a suitable rack in a pan of water at 37-40 C.

A seed rat carrying the trypanosomes for the tests is selected whose blood as examined microscopically with a 1/6 objective and No. 4 eyepiece (Leitz) by placing a drop from the tail upon a cover glass and suspending it over a concave slide, shows a large number of active trypanosomes. One cubic centimeter of a 1% solution of sodium citrate in normal salt or Ringer's solution is placed in a test tube and warmed; sufficient blood is then secured from the heart or tail of the seed rat to give an emulsion of trypanosomes which, when examined as described above, will show at least 10 trypanosomes in each field and particularly at the margin of the drop. The sodium citrate is added to prevent coagulation of the blood, but more than a 2% solution is not advisable. This blood trypanosome emulsion is kept warm by placing the tube in the water bath.

Slides having two or more concavities are prepared and ringed with vaselin. With a 3-mm. platinum loop a drop of a dilution of the chemical is placed in the center of a cover glass; the wire is heated and cooled and a drop of the blood emulsion added and mixed in exactly the same manner as in preparing a microscopic agglutination test. The cover glass is then adjusted to a concavity in the slide and carefully sealed with vaselin. The dilution of chemical now acting upon the trypanosomes is doubled. The time when blood and chemical are mixed is carefully noted, and the slide placed on a warm stage of the microscope or in a small oven heated to 37-40 C. placed alongside of the microscope.

¹ This report is to be published later.

² This report is to be published later.

³ Jour. Infect. Dis., 1916, 18, p. 247.

A number of preparations are made as rapidly as possible with the aid of an assistant, including at least 2 controls prepared by mixing a drop of trypanosome emulsion with a drop of warm normal salt or Ringer's solution.

It is advisable to keep the solutions of chemical, the trypanosome emulsions, and the finished slides at body temperature in order to avoid the effect of chilling on the trypanosomes.

At definite intervals, ranging, for example, from 5 or 10 minutes to an hour or longer from the time of exposure of the trypanosomes to the chemical the slides are examined microscopically and compared with the controls.

As a general rule, the controls show active trypanosomes for at least 1 to 2 hours or longer; after this time the movements may become sluggish or cease entirely in a few of the parasites. For the purpose of avoiding error it is our custom to terminate the experiment at the end of 1 hour.

In case the chemical under study exerts an influence on the trypanosomes, the parasites first lose their to and fro movements and remain in one position with constant vibratile movement. Later the latter movement becomes more and more sluggish, finally ceasing altogether, and certain structural changes occur. These effects are best determined by inspecting the trypanosomes at the margin of the drop; before motility is regarded as completely destroyed, a number of fields should be carefully inspected.

With practice and assistance a large number of preparations may be made in a short time. It is our custom to permit at least one-half minute for the preparation of each cover glass. As a general rule it is advisable to examine a preparation within 10 minutes of the time when chemical and trypanosomes are mixed; strong dilutions of a trypanocidal substance, as arsenobenzol, produce an effect within a few minutes.

The results of a few experiments with various substances conducted after this method are shown in Tables 1, 2, and 3.

Of particular interest are the effects noted with arsenobenzol, which has been claimed to be relatively inert *in vitro*, and with bichlorid of mercury, which is so toxic that a sufficient dose to influence trypanosomiasis in the living animal cannot be given.

This method is open, however, to some degree of error. The results of experiments with the same substance at different times with the same strain of trypanosomes have been found to vary, hence the results should be interpreted as showing approximately the relative trypanocidal activity of different substances judged solely by their influence on the motility and appearance of the parasites; loss of motility, however, is not necessarily an indication of the death of a parasite.

THE IN-VITRO-VIVO CENTRIFUGE METHOD

In this method the trypanosomes are exposed for a certain interval to a definite dilution of chemical, are then removed and washed by centrifugation, and injected into the peritoneal cavities of white rats to determine whether or not their destruction has been brought about. The test thus becomes a combined *in-vitro-vivo* test.

TABLE 1
 TRYPANOCIDAL ACTIVITY OF EMETIN

Dilutions	Minutes of Time Required to Kill all Trypanosomes	
	T. Lewisii	T. Equiperdum
1 : 100.....	3 to 10	17 to 55
1 : 200.....	10	13 to 32
1 : 400.....	12	11 to 19
1 : 800.....	14	21 to 30
1 : 1600.....	15 to 18	30 to 41
1 : 3200.....	20 to 40	40 to 50
1 : 6400.....	25 to 45	60 to 72
1 : 12800.....	30 to 70	100 to 130
1 : 25600.....	60 to 90	125 to 140
1 : 51200.....	70 to 120	150 to 190
1 : 102400.....	80 to 140	

TABLE 2
 MICROSCOPIC TRYPANOCIDAL TEST WITH BICHLORID OF MERCURY AND T. EQUIPERDUM

Dilutions	Loss of Motility After Exposure, in Minutes								
	8	12	16	20	24	30	40	50	60
1 : 100	—								
1 : 150	—								
1 : 200	—								
1 : 300	—								
1 : 400	—								
1 : 600	—								
1 : 800	—								
1 : 1200	—								
1 : 1600	—								
1 : 2400	—								
1 : 4800	+	—							
1 : 6400	+								
1 : 12800	+	+	+	+	—				
1 : 25600	+	+	+	+	—				
1 : 50000	+	+	+	+	+	—			
1 : 100000	+	+	+	+	+	+	+	+	+
1 : 200000	+	+	+	+	+	+	+	+	+
Controls	+	+	+	+	+	+	+	+	+

+ = motility; — = loss of motility.

TABLE 3
 MICROSCOPIC TRYPANOCIDAL TEST WITH ARSENOBENZOL AND T. EQUIPERDUM

Dilutions	Loss of Motility After Exposure, in Minutes								
	8	12	16	20	24	30	40	50	60
1 : 100	—								
1 : 150	+	—	—						
1 : 200	+	+	—						
1 : 300	+	+	+	—					
1 : 400	+	+	+	—					
1 : 600	+	+	+	+					
1 : 800	+	+	+	+	—				
1 : 1200	+	+	+	+	—				
1 : 1600	+	+	+	+	+	—			
1 : 2400	+	+	+	+	+	—			
1 : 4800	+	+	+	+	+	—			
1 : 6400	+	+	+	+	+	+			
1 : 12800	+	+	+	+	+	+	—		
1 : 25600	+	+	+	+	+	+	+	+	—
1 : 50000	+	+	+	+	+	+	+	+	+
1 : 100000	+	+	+	+	+	+	+	+	+
1 : 200000	+	+	+	+	+	+	+	+	+
Controls	+	+	+	+	+	+	+	+	+

+ = motility; — = loss of motility.

A strain of trypanosomes which is pathogenic for white rats should be used, such as *T. equiperdum* or *T. brucei*; the former, which usually kills the host in from 4 to 7 days after inoculation, has been found uniformly satisfactory.

Technic.—A seed rat is selected whose blood, as examined in a drop from the tail, shows the presence of a large number of trypanosomes. If it is desired to expose approximately known numbers of trypanosomes to definite dilutions and amounts of chemical, as is advisable in comparative tests, the trypanosomes may be counted by the method of Kolmer⁴ and the approximate number per cubic centimeter of blood determined. Experience has shown us that it is advisable to work with an emulsion containing at least 100,000,000,000 trypanosomes per cubic centimeter.

Blood is drawn from the heart or from the vessels in the neck into a measured amount of a warm 1% solution of sodium citrate in normal salt solution until the desired dilution is obtained. If an actual count is not made, the blood of a heavily infected rat may be diluted with 3 parts of this solution.

Dilutions of the chemical in warm normal salt solution and in amounts of 0.5 c.c. are prepared in centrifuge tubes and placed in a water bath at 40 C.; to each tube and several controls of salt solution alone, is now added 0.5 c.c. of the blood trypanosome emulsion and the tube gently shaken. After an exposure of 15 minutes, 10 or 15 c.c. of warm salt solution are added to each tube to dilute the chemical and wash the trypanosomes, and the tubes are centrifugated at high speed.

To insure uniform success it is imperative to avoid the extreme chilling consequent upon rapid centrifugation. To this end the centrifuge cup should be large and filled with water at 42 C. Even under these circumstances chilling may occur and cause irregular results.

The supernatant fluid is now removed; 1 c.c. of warm salt solution is added to each tube, and the sediment of blood cells and trypanosomes is mixed and injected into the peritoneum of a white rat.

Daily examinations are made of the blood from the tail and the trypanocidal influence of the chemical judged according to the time elapsing before trypanosomes appear in the peripheral blood and the duration of life thereafter as compared with that of the controls.

The results of an experiment conducted with arsenobenzol are shown in Table 4.

In conducting this test it is necessary to use large numbers of trypanosomes and to avoid chilling as much as possible. The rats should be kept under observation for at least 2 weeks before being considered sterile, as trypanosomes may appear in the peripheral blood during this time.

THE COMBINED IN-VITRO-VIVO METHOD

In this method equal parts of varying dilutions of the chemical under study are mixed with blood trypanosome emulsion and kept at 37-40 C., when the whole or a part of each is injected intraperi-

⁴ Jour. Infect. Dis., 1915, 17, p. 79.

TABLE 4

THE IN-VITRO-VIVO CENTRIFUGE METHOD (TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL FOR *T. EQUIPERDUM*)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 320,000,000.

Approximate number of trypanosomes in 1 c.c. of diluted blood: 106,000,000.

Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 40 C.

Duration of exposure: 15 minutes + centrifugation.

Approximate amount of chemical in contact with the trypanosomes: given in the table.

Approximate number of trypanosomes injected: 53,000,000.

Rat	Weight, gm.	Dilutions	Amount, gm.	Results of Daily Examinations of Tail Blood									
				1	2	3	4	5	6	7	8	9	10
1	90	1:1000	.001	—	—	—	—	—	—	—	—	—	—
2	83	1:2000	.0005	—	—	—	—	—	—	—	—	D	—
3	84	1:4000	.00025	—	—	—	—	—	—	—	—	—	—
4	72	1:8000	.000125	—	—	—	—	—	—	—	—	—	D
5	80	Control	0	Few	Few	Few	+	++	++	D	—	—	—
6	91	Control	0	—	—	—	Few	+++	D	—	—	—	—
7	97	Control	0	—	Few	Few	+	+	+	++	++	D	—
8	82	Control	0	—	Few	+	+++	++++	++++	D	—	—	—

toneally into white rats to determine the degree of trypanocidal activity. As a part of the drug is injected, the action of the drug is both in vitro and in vivo and due care must be exercised against the administration of lethal doses of the drug.

Technic.—A series of dilutions of the drug under study is prepared in sterile normal salt or Ringer's solution in small sterile test tubes (1×6 cm.) and in amounts of 0.5 c.c. The series is placed in a water bath at 37-40 C. to be warmed to body temperature.

A rat heavily infected with *T. equiperdum* or *T. brucei* is selected, and a count made of the number of trypanosomes per cubic centimeter of blood.

Blood is then secured from the heart or vessels of the neck in sufficient sterile 1% solution of sodium citrate in normal salt or Ringer's solution warmed to 40 C. to give the desired dilution. Each cubic centimeter of this suspension should contain at least 20,000,000 trypanosomes. After a count has been made and the amount of dilution calculated, it is good practice to place the diluent in a small graduated cylinder and bleed the rat from the neck into the cylinder by means of a sterile funnel until the desired amount of blood has been added. This mixture is then gently shaken and afterward kept at a temperature of 37-40 C. in a water bath.

At an appointed time 0.5 c.c. of the trypanosome emulsion is added to each tube containing the solution of chemical and to a series of controls containing 0.5 c.c. of sterile normal salt or Ringer's solution. Each tube is gently shaken and then kept in the water bath at 37-40 C. for 15 minutes. The trypanosomes should be added to the chemical at intervals of one-half minute, which permits sufficient time for the injection of each.

At the end of the period of exposure 0.5 c.c. of the blood trypanosome chemical mixture is injected into the peritoneum of a rat. One-half minute is assigned for each injection. A 1-c.c. Record syringe fitted with a 22 platinum iridium needle is well adapted for the injections. After each injection there is sufficient time to wash out the syringe and needle several times with hot normal salt solution conveniently placed near the operator.

TABLE 5

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY AND ARSENOBENZOL FOR *T. EQUIPERDUM*)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 240,000,000.

Dilution of blood contains approximately per cubic centimeter: 120,000,000.

Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 39 C.

Duration of exposure: 15 minutes.

Amount of chemical trypanosome mixture injected: 0.5 c.c.

Approximate number of trypanosomes injected: 30,000,000.

Approximate amount of chemical injected: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Bichlorid Hg	1	70	1 : 5000	.0002
	2	75	1 : 10,000	.0001
	3	122	1 : 15,000	.000066
	4	130	1 : 20,000	.00005
	5	105	1 : 30,000	.000033
	6	107	1 : 40,000	.000025
	7	68	1 : 80,000	.0000125
Arsenobenzol.....	8	116	1 : 200	.005
	9	90	1 : 400	.0025
	10	106	1 : 800	.00125
	11	100	1 : 1600	.00062
	12	114	1 : 3200	.00031
	13	89	1 : 6400	.00015
	14	80	1 : 12,800	.000075
Controls.....	15	107	0	0
	16	92	0	0
	17	94	0	0
	18	90	0	0

Each rat is then kept under observation for at least 2 weeks and the blood examined daily for trypanosomes. In this manner the degree of trypanocidal activity may be determined according to the time when trypanosomes appear and the duration of life as compared with that of the controls.

The results of a few of the series of experiments conducted with various substances are shown in Tables 5 to 8.

The viability of the trypanosomes under the conditions of this technic is indicated in Table 9, which gives the results of one of several experiments to determine this point. The uniform success with which the controls infect rats also indicates that *T. equiperdum* withstands very well an exposure in normal salt solution at 37 to 40 C. for at least 15 minutes. As shown in these and other experiments, the 15-minute period of exposure does not materially lower the viability or virulence of these trypanosomes.

As a part of the chemical is injected into the peritoneum of the rat, its trypanocidal activity is probably continued in vivo. To test the degree of trypanocidal activity under these conditions, a number of experiments were conducted with the same solutions of chemical and trypanosome emulsions, in some of which the two emulsions

TABLE 6

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY
AND ARSENOBENZOL FOR *T. EQUIPERDUM*)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood:
312,000,000.

Dilution of blood contains approximately per cubic centimeter: 104,000,000.

Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 38 C.

Duration of exposure: 15 minutes.

Amount of chemical trypanosome mixture injected: 0.5 c.c.

Approximate number of trypanosomes injected: 26,000,000.

Approximate amount of chemical injected: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Bichlorid of mercury.	1	100	1 : 10,000	.0001
	2	102	1 : 15,000	.000066
	3*	110	1 : 20,000	.00005
	4	115	1 : 30,000	.000033
	5	67	1 : 40,000	.000025
	6	94	1 : 60,000	.000016
	7	89	1 : 80,000	.0000125
	8	85	1 : 120,000	.0000083
	9	90	1 : 160,000	.0000062
	10	102	1 : 240,000	.00000416
Arsenobenzol.....	11	91	1 : 400	.0025
	12	80	1 : 600	.0015
	13	55	1 : 800	.00125
	14	71	1 : 1200	.00083
	15	113	1 : 1600	.00062
	16	89	1 : 2400	.00041
	17	84	1 : 4800	.000208
	18	105	1 : 9600	.000104
	19	91	1 : 19,200	.000052
	20	88	1 : 40,000	.000026
Control.....	21	87	0	0
	22	93	0	0
	23	77	0	0

TABLE 7

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF VARIOUS MERCURIALS
AND PHENOL FOR *T. EQUIPERDUM*)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood:
256,000,000.

Dilution of blood contains approximately per cubic centimeter: 64,000,000.

Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 40 C.

Duration of exposure: 15 minutes.

Amount of chemical trypanosome mixture injected: 0.5 c.c.

Approximate number of trypanosomes injected: 16,000,000.

Approximate amount of chemical injected: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Bichlorid Hg.....	1	105	1 : 2500	.0004
	2	160	1 : 5000	.0002
	3	102	1 : 10,000	.0001
	4	128	1 : 20,000	.00005
	5	100	1 : 40,000	.000025
Salicylate Hg.....	6	132	1 : 2500	.0004
	7	102	1 : 5000	.0002
	8	85	1 : 10,000	.0001
	9	94	1 : 20,000	.00005
	10	103	1 : 40,000	.000025
Calomel.....	11	100	1 : 2500	.0004
	12	82	1 : 5000	.0002
	13	85	1 : 10,000	.0001
	14	89	1 : 20,000	.00005
	15	76	1 : 40,000	.000025
Phenol.....	16	100	1 : 100	.01
	17	87	1 : 200	.005
	18	77	1 : 400	.0025
	19	87	1 : 800	.00125
	20	93	1 : 1600	.000625
Control.....	21	76	0	0
	22	70	0	0
	23	58	0	0
	24	78	0	0

TABLE 8

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF VARIOUS NEW COMPOUNDS OF MERCURY FOR *T. EQUIPERDUM*)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 192,000,000.

Dilution of blood contains approximately per cubic centimeter: 48,000,000.

Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 37 C.

Duration of exposure: 15 minutes.

Amount of chemical trypanosome mixture injected: 0.5 c.c.

Approximate number of trypanosomes injected: 12,000,000.

Approximate amount of chemical injected: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Bichlorid.....	1	78	1 : 2500	.0004
	2	70	1 : 5000	.0002
	3	95	1 : 10,000	.0001
	4	95	1 : 20,000	.00005
	5	108	1 : 40,000	.000025
No. 41.....	6	78	1 : 2500	.0004
	7	92	1 : 5000	.0002
	8	80	1 : 10,000	.0001
	9	97	1 : 20,000	.00005
	10	87	1 : 40,000	.000025
No. 2.....	11	101	1 : 2500	.0004
	12	103	1 : 5000	.0002
	13	85	1 : 10,000	.0001
	14	89	1 : 20,000	.00005
	15	90	1 : 40,000	.000025
No. 4.....	16	78	1 : 2500	.0004
	17	92	1 : 5000	.0002
	18	82	1 : 10,000	.0001
	19	115	1 : 20,000	.00005
	20	88	1 : 40,000	.000025
	21	83	0	0
	22	90	0	0
	23	70	0	0
	24	80	0	0

TABLE 9

THE VIABILITY OF *T. EQUIPERDUM* IN THE COMBINED IN-VITRO-VIVO METHOD

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 368,000,000.

Dilution of blood contains approximately per cubic centimeter: 92,000,000.

Temperature of medication: 37 C.

Duration of exposure: stated in the table.

Amount of normal-salt-solution trypanosome mixture injected: 0.5 c.c.

Approximate number of trypanosomes injected: 23,000,000.

Rat	Weight, gm.	Exposure
1.....	110	At once
2.....	100	15 min.
3.....	62	30 min.
4.....	102	45 min.
5.....	99	60 min.
6.....	106	1½ hr.
7.....	100	2 hr.

TABLE 10

THE COMBINED IN-VITRO-VIVO METHOD COMPARED WITH AN IN-VITRO TEST (TRYPANOCIDAL ACTIVITY OF SALICYLATE OF MERCURY IN ALKALINE SOLUTION)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 320,000,000.

Dilution of blood contains approximately per cubic centimeter: 80,000,000.

Proportion of solution of chemical to trypanosome emulsion in the in-vitro-vivo test: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 37 C.

Duration of exposure: 15 minutes.

Amount of chemical solution injected alone in the in-vivo test: 1 c.c.

Approximate number of trypanosomes injected in both tests: 20,000,000.

Approximate amount of chemical injected in both tests: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Mixed.....	1	90	1 : 2500	.0004
	2	103	1 : 5000	.0002
	3	95	1 : 10,000	.0001
	4	62	1 : 20,000	.00005
Separate.....	5	95	1 : 2500	.0004
	6	70	1 : 5000	.0002
	7	70	1 : 10,000	.0001
	8	82	1 : 20,000	.00005
Alkali.....	9	81	Undiluted	
	10	62	1 : 2	
35 Min.	11	80	Control	
	12	75	Control	
	13	79	Control	
	14	71	Control	

TABLE 11

THE COMBINED IN-VITRO-VIVO METHOD COMPARED WITH AN IN-VIVO TEST (TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 128,000,000.

Dilution of blood contains approximately per cubic centimeter: 32,000,000.

Proportion of solution of chemical to trypanosome emulsion in the in-vitro-vivo test: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 38 C.

Duration of exposure: 15 minutes.

Amount of chemical solution injected alone in the in-vivo test: 0.5 c.c.

Amount of chemical trypanosome mixture injected in the vitro-vivo test: 1 c.c.

Approximate number of trypanosomes injected in both tests: 8,000,000.

Approximate amount of chemical injected in both tests: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Mixed.....	1	102	1 : 2000	.0005
	2	101	1 : 4000	.00025
	3	120	1 : 8000	.000125
	4	102	1 : 16,000	.000062
Separate.....	5	105	1 : 2000	.0005
	6	96	1 : 4000	.00025
	7	135	1 : 8000	.000125
	8	136	1 : 16,000	.000062
	9	103	Control	0
	10	92	Control	0
	11	88	Control	0
	12	102	Control	0

TABLE 10—Continued

THE COMBINED IN-VITRO-VIVO METHOD COMPARED WITH AN IN-VITRO TEST (TRYPANOCIDAL ACTIVITY OF SALICYLATE OF MERCURY IN ALKALINE SOLUTION)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 320,000,000.

Dilution of blood contains approximately per cubic centimeter: 80,000,000.

Proportion of solution of chemical to trypanosome emulsion in the in-vitro-vivo test:

Temperature of medication: 37 C.

Duration of exposure: 15 minutes.

Amount of chemical solution injected alone in the in-vivo test: 1 c.c.

Approximate number of trypanosomes injected in both tests: 20,000,000.

Approximate amount of chemical injected in both tests: stated in the table.

Results of Daily Examinations of Tail Blood												
1	2	3	4	5	6	7	8	9	10	11	12	13
Hemolyzed												
—	—	—	—	—	—	—	—	—	—	—	Few	D
—	—	—	Few	Few	D	—	—	—	—	—	Few	D
—	—	—	—	—	—	—	D					
—	—	Few	+	+++	++++	D						
—	—	Few	+	+++	++++	D						
—	Few	+++	++++	++++	++++	D						
—	—	—	—	—	D	—	—	—	—	—	Few	D
—	—	—	—	—	—	—	—	—	—	—		
—	Few	+	++++	++++	D							
Few	Few	+	++++	++++	++++	D						
—	—	+	+	+	D							
Few	Few	+	+++	++++	++++	D						

TABLE 11—Continued

THE COMBINED IN-VITRO-VIVO METHOD COMPARED WITH AN IN-VIVO TEST (TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood:
128,000,000.

Dilution of blood contains approximately per cubic centimeter: 32,000,000.

Proportion of solution of chemical to trypanosome emulsion in the in-vitro-vivo test:
0.5 c.c. + 0.5 c.c.

Temperature of medication: 38 C.

Duration of exposure: 15 minutes.

Amount of chemical solution injected alone in the in-vivo test: 0.5 c.c.

Amount of chemical trypanosome mixture injected in the vitro-vivo test: 1 c.c.

Approximate amount of chemical injected in both tests: stated in the table.

Results of Daily Examinations of Tail Blood											
1	2	3	4	5	6	7	8	9	10	11	12
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	Few	++	+++++	+++++	D	—
—	—	—	—	—	Few	+++	+++++	+++++	+++++	D	—
—	—	—	—	—	—	—	++	+++++	+++++	+	—
—	—	—	—	—	—	—	—	+	+++++	+++++	D

TABLE 12

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL IN THE IN-VIVO AND THE COMBINED IN-VITRO-VIVO METHODS

Method	Rat	Weight, gm.	Dose
Routine In-Vivo.....	1*	116	.004†
	2	82	.003
	3	83	.002
	4	102	.001
	5	110	Control
	6	122	Control
Combined In-Vitro-Vivo.....	7	80‡	.00164§
	8	71	.00082
	9	89	.00041
	10	84	.000208
	11	105	.000104
	12	91	.000052
	13	88	.000026
	14	85	.000013
	15	87	Control
	16	93	Control

* Rats infected by intraperitoneal injection of 500,000 T. equiperdum. This is a greater infection than usual in our routine tests and with a resistant strain of T. equiperdum—facts which account for the failure of the 0.002-gram dose of arsenobenzol to sterilize.*

‡ Kolmer, Schamberg and Raiziss: Jour. Infect. Dis., 1917, 20, p. 35.

TABLE 13

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF COMPOUND NO. 85-15 (A DERIVATIVE OF ARSENOBENZOL) IN THE IN-VIVO AND COMBINED IN-VITRO-VIVO METHODS

Method	Rat	Weight, gm.	Dose		
				1	2
Routine In-Vivo.....	1*	87	.008†	—	—
	2	144	.007	—	—
	3	122	.006	—	—
	4	102	.004	—	—
	5	86	.002	—	+
	6	110	Control	—	+
	7	122	Control	—	+
Combined In-Vitro-Vivo....	8	80‡	.01§	—	—
	9	95	.005	—	—
	10	100	.0025	—	—
	11	110	.00125	—	—
	12	90	Control	—	—
	13	65	Control	Few	+
	14	70	Control	—	—

* Rats infected by intraperitoneal injection with 500,000 T. equiperdum.

† Dose of Compound No. 85-15 per 100 gm. of body weight; injected intravenously 24 hours after infection.

TABLE 14

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY IN THE IN-VIVO AND THE COMBINED IN-VITRO-VIVO METHODS

Method	Rat	Weight, gm.	Dose
Routine In-Vivo.....	1*	49	.0003†
	2	66	.0002
	3	55	.0001
	4	70	.00005
	5	80	Control
	6	65	Control
Combined In-Vitro-Vivo.....	7‡	78	.0004§
	8	70	.0002
	9	95	.0001
	10	95	.00005
	11	108	.000025
	12	90	Control
	13	70	Control

* Rats infected by intraperitoneal injection with 120,000 T. equiperdum 24 hours before the intravenous administration of bichlorid of mercury.

† Doses of bichlorid of mercury per 100 gm. of body weight.

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL IN THE IN-VIVO AND THE COMBINED IN-VITRO-VIVO METHODS

† Dose of arsenobenzol per 100 gm. of body weight; injected intravenously 24 hours after injection.
‡ Combined in-vitro-vivo method conducted with 26,000,000 trypanosomes.
§ Amount of arsenobenzol to which the trypanosomes had been exposed for 15 minutes in vitro.

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF COMPOUND NO. 85-15 (A DERIVATIVE OF ARSENOBENZOL) IN THE IN-VIVO AND COMBINED IN-VITRO-VIVO METHODS

§ Amount of Compound No. 85-15 to which the trypanosomes had been exposed for 15 minutes in vitro.

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY IN THE IN-VIVO AND THE COMBINED IN-VITRO-VIVO METHODS

‡ Combined in-vitro-vivo method conducted with 12,000,000 *T. equiperdum*.
§ Amount of bichlorid of mercury to which the trypanosomes had been exposed for 15 minutes in vitro.
¶ Death probably due to toxicity of mercury.

¶ Death probably due to toxicity of mercury.

As previously stated, the routine in-vivo test is conducted by infecting rats with intraperitoneal injections of *T. equiperdum*, averaging 120,000 trypanosomes per animal. Twenty-four hours later the drug is administered intravenously according to the body weight of each rat, each dose being dissolved in 1 c.c. of sterile distilled water.

The combined in-vitro-vivo tests were conducted by exposing about 25,000,000 to 50,000,000 *T. equiperdum* contained in 0.5 c.c. diluted blood to varying amounts of the chemical dissolved in 0.5 c.c. of normal salt solution.

The results observed with both methods with several substances are shown in Tables 12, 13, and 14 as examples of the results of these tests.

In Table 15 is given a summary of the results obtained with both tests with a number of new compounds prepared in the course of our chemotherapeutic researches, and several well-known bactericides.

TABLE 15

COMPARATIVE RESULTS WITH THE ROUTINE IN-VIVO AND THE COMBINED IN-VITRO-VIVO METHODS

Substance*	Routine In-Vivo Test		Combined In-Vitro-Vivo Test	
	Dose per 100 gm. Body Weight	Results (over period of 15 days)	Dose	Results (over period of 15 days)
Arsenobenzol0015	Trypanocidal	.000052	Trypanocidal
85-15 (a derivative of arsenobenzol)006	Trypanocidal	.00125	Trypanocidal
39 (a mercurial compound of naphthaline series)0003†	Not trypanocidal	.00025	Trypanocidal
42 (a compound of guaiacol-mercury)0004†	Not trypanocidal	.00025	Trypanocidal
47 (a derivative of guaiacol) ..	.002†	Not trypanocidal	.001	Trypanocidal
60 (an organic compound of copper)0009†	Not trypanocidal	.0005	Trypanocidal
64 (copper salicylate)001†	Not trypanocidal	.001	Trypanocidal
Copper sulfate001†	Not trypanocidal	.0025	Trypanocidal
Phenol01†	Not trypanocidal	.005	Trypanocidal
Bichlorid of mercury0003†	Not trypanocidal	.000033	Inhibitory for 4 days
2 (a mercury compound of the naphthol series)0003†	Not trypanocidal	.00005	Trypanocidal
41 (a mercury compound)0002†	Not trypanocidal	.0004	Inhibitory for 8 days

* The chemistry of these new compounds will be given in a later communication.

† About the lethal dose for rats by intravenous injection per 100 gm. of body weight.

As shown in the tables the results obtained with the combined in-vitro-vivo method are generally clear-cut and decisive.

At times the animals are injected intraperitoneally with a lethal dose of the drug; in conducting the combined in-vitro-vivo method these doses are to be avoided.

As shown in Table 12 arsenobenzol, or salvarsan, possesses a very

high degree of trypanocidal activity in the combined in-vitro-vivo method. As shown in Tables 3, 4, 5, and 6 this activity is certainly, in part at least, due to the influence of arsenobenzol upon trypanosomes in vitro.

The combined in-vitro-vivo method always indicates a smaller trypanocidal dose for a compound than the routine in-vivo test.

Substances which in amounts just sublethal, exert no appreciable influence upon trypanosomes in vivo, in the combined in-vitro-vivo method either are definitely trypanocidal or in a certain dosage inhibit the multiplication of trypanosomes and their appearance in the peripheral blood for a certain number of days. In this manner results with the combined in-vitro-vivo method frequently yield information of value and serve as a guide for further researches with a particular and promising therapeutic compound or group of compounds.

CONCLUSIONS

Trypanocidal tests in vitro have been found of distinct value in chemotherapeutic researches in experimental trypanosomiasis.

Substances exerting a profound trypanocidal activity in vitro are likely to prove trypanocidal in vivo, provided the drug is sufficiently nontoxic to be administered in adequate dosage.

With the combined in-vitro-vivo method described herein, it has been found possible to detect the trypanocidal activity of new compounds which were without effect in vivo in amounts but slightly less than the sublethal dose.

By the methods described herein arsenobenzol, or salvarsan, has been shown to possess a high trypanocidal activity in vitro.

In-vitro methods have also demonstrated a trypanocidal activity on the part of mercurials which is not apparent in the in-vivo tests.

FURTHER OBSERVATIONS ON THE ADAPTATION OF PARASITIC MICROORGANISMS TO A LOWERED OXYGEN TENSION *

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We have already published notes which tend to establish the generalization that many, if not all, endoparasites become adapted to a tension of oxygen below the atmospheric.¹ The following observations furnish further proof:

Partial-Tension Filaria.—Embryo filaria in the blood of a Carolina bullfrog survived for a number of days, at least 9, under partial oxygen tension, while they died within 24 hours under aerobic conditions. In the partial-tension culture there appeared a culture of flagellates resembling the cultural forms of a trypanosome, while no such organisms appeared in the aerobic culture.² We have suggested that this partial oxygen requirement is probably an important factor in the life history of many embryo nematodes, cestodes, and trematodes. It may, for example, help to explain why the embryos of *Ankylostoma* and *Necator* thrive best in a sandy soil.

Partial-Tension Herpetomonads.—We have made aerobic and partial-tension cultures (in the water of syneresis on slants of +0.5 Martin's medium containing pleuritic and ascitic fluid) of herpetomonads from the intestinal tract of the green bottle fly. The herpetomonads survived in an actively motile condition for 24 hours at 28 C., but after that the accompanying bacteria overgrew them entirely. The interesting point to record here is the relation of oxygen to the motility of the herpetomonads in these two different cultures: When mounted under a coverslip from the aerobic culture the herpetomonads remained motile only at the edges of the preparation or at the periphery of a bubble of air; in a similar preparation from the partial-tension culture the reverse obtained.

* Received for publication October 11, 1916.

¹ Jour. Infect. Dis., 1916, 19, p. 288.

² Lancet Clinic, 1916, 116, p. 1.

Partial-Tension Streptococcus from a Microaerophile-Aerophobe Strain.—The history of the isolation of the microaerophile-aerophobe strain was as follows: About 3 c.c. of prostatic fluid were "collected aseptically" from a case of low-grade prostatitis accompanied occasionally by mild attacks of sciatica. (It is not claimed here that there was any connection between the isolated organism and the condition of the patient. The organism might even have been a transient inhabitant of the urethra. In fact an attempt to isolate the same organism 2 weeks later failed.) The prostatic fluid was diluted with 10 c.c. of autoclaved distilled water and centrifugated at high speed for 30 minutes. The sediment was planted on slants of $+0.5$ Martin's medium, containing sterile ascites fluid, and incubated at 37 C., aerobically, at partial tension, and anaerobically. In 48 hours there was no aerobic growth (nor did any appear subsequently), while the partial-tension slant showed a number of very minute colonies, which a few days later attained the size and appearance of the colonies on the anaerobic slant. The anaerobic slant in 48 hours showed about 15 colonies, 3-4 mm. in diameter, composed of very long chains of streptococci.

One of the colonies on the anaerobic slant (which appeared pure macroscopically and microscopically) was subcultured to a tube of the same medium and incubated anaerobically at 37 C. Three days later the long chains in the water of syneresis of this anaerobic slant were thoroughly mixed with 10 c.c. of autoclaved salt solution and about 2 c.c. of this emulsion were planted with a sterile capillary pipet throughout the various portions of a deep column (10 cm.) of $+0.5$ Martin's medium containing an equal volume of sterile ascites fluid. After 24 hours' growth at 37 C. the tube presented the appearance shown in Fig. 1. That is, the growth was in 2 widely separated zones: an upper, 5 mm. broad, beginning about 1 mm. below the surface, and a deep zone about 10 mm. broad and sharply separated from the bottom of the test tube by a layer of colony-free agar 5 mm. high. The agar between the zones was entirely free of visible growth except one rather large colony as represented in Fig. 1. This tube maintained this appearance during 6 days' incubation at 37 C., except that some of the upper colonies in both zones increased in size, being comparatively isolated in distribution.

A similar phenomenon has been recorded by Wittneben.³ While reading his article sometime previously, we were inclined to believe that Wittneben had

³ Centralbl. f. Bakteriöl., I. O., 1907, 44, p. 96.

been working with a mixed culture. It seemed extremely improbable to us that the culture of a single species, all the individuals of which were grown under the same conditions, could be composed of descendants adapted to such widely varying oxygen requirements. However, the following observations have reversed our preconceived ideas on this subject. But before we take up the study of the oxygen requirements of the cocci in these two widely separated layers of growth, we shall describe briefly the microscopic appearance of the colonies in the upper, and deeper layers. Some of the colonies appeared, under the hand lens, to be well defined and lenticular, like staphylococcus colonies, and when a mixture of these colonies was crushed under a coverglass, it was seen that some were composed of cocci in pairs and masses like staphylococci while most of the colonies were composed of streptococci. All of these retained the stain in Gram's method. The colonies in the deepest portion of the deep layer seemed to be composed entirely of long-chained streptococci, but one of the larger colonies near the upper limits of the deep layer was composed of cocci in chains, and pairs, and massed like staphylococci.

Now, as we did not encounter the staphylococcus arrangement in any of our subsequent subcultures on slanted media, whereas lenticular colonies frequently appeared among the others in the deep subcultures, we are inclined to believe that it will be worth while to investigate the variations in the mass grouping of bacteria, for this character may have been given too important a position in classification.

After the deep tube culture (Fig. 1) had been kept for 6 days at 37 C. the following studies were made:

(a) The upper layer: A block of colonies was cut out and ground up with sterile salt solution. A few drops of this suspension were allowed to flow over the surface of a slant of Martin's pleuritic medium and this was incubated at 37 C. after being attached to a freshly inoculated slant of *B. subtilis*. In 24 hours about 75 colonies, 0.25-2 mm. in diameter, were present on the surface, and the water of syneresis was filled with flocculi composed of long chains.

(b) The deep layer: After the tube had been cut at the level of the lowest colonies, a block was cut out at the point marked \times (Fig. 1). This was planted in the same way as described under (a) except that here both partial-tension and anaerobic cultures were made. In 24 hours the growth on the partial-tension slant resembled that described under (a). The anaerobic colonies were larger, 2-4 mm.

Transplants were made from well-isolated colonies on each of these slants to slants of +0.5 Martin's pleuritic medium and incubated at 37 C. under the corresponding oxygen tensions.

Also, when the slant subcultures (mixed colony subcultures) described under (a) and (b), were 48 hours old, the growth in the water of syneresis was emulsified with sterile salt solution and planted in deep tubes of +0.5 Martin's medium containing an equal volume

of ascites fluid. Of course in all such transfers separate sterile pipets were used. The anaerobic culture from the deep layer was designated (a), the partial-tension culture from the same (b), and the partial-tension from the upper layer (c). The results are shown in Figs. a, b, and c. That is, the anaerobic culture from the deep layer (a) yielded numerous relatively anaerobic colonies, 1-2 mm. in diameter, extending from 12 mm. below the surface to the bottom of the test tube; and extending from just beneath the surface to a depth of 5 mm. there were very numerous minute colonies. The growths in (b) and (c) were very much like those in (a), but the number of deep anaerobic colonies diminished progressively. During 7 days' incubation at 37 C. these growths remained much the same except that very fine colonies appeared throughout. No growth at any time occurred on the surface.

The single-colony transfers referred to under (b) were not tested for their oxygen relationships until after 7 days' incubation at 37 C. These were designated a', b', and c' to correspond with a, b, and c. The growths in the water of syneresis were planted in the same way, the same batch of medium and the same batch of ascites fluid being used as in the case of a, b, and c. The results after 24 hours at 37 C. are shown in Figs. a', b', and c'. For example:

(a') It will be noted that there were very few minute colonies near the surface; a layer of colonies 0.25 mm. in diameter, which was 6 mm. broad, extended from 2 mm. below the surface; then a space of 2 mm. free of colonies; then scattered large colonies, 1-2 mm. in diameter, extending to the bottom of the test tube, and between these a few smaller colonies.

(b') Here there was a layer of very numerous minute colonies extending from just beneath the surface to a depth of 5 mm. The rest of the tube was free of growth, except for 6 scattered colonies 1-2 mm. in diameter and about a dozen scattered minute colonies.

(c') This showed still further tendency to partial-tension growth in that only one deep colony, 0.5 mm. in diameter, developed.

Now we should like to remark right here that we entertained the idea that up to the point where Experiments a, b, and c were performed we might have been carrying over a mixed culture of a microaerophile and an aerophobe. But the single-colony subculture tests we think indicate that organisms in a pure culture may vary considerably and that if maintained at a certain tension for a sufficient length of time.

most of the individuals tend to adapt their respiratory mechanism to that tension. That the transformation may have to be brought about gradually and may be accompanied by the development of a large number of related types, we think extremely probable. We also

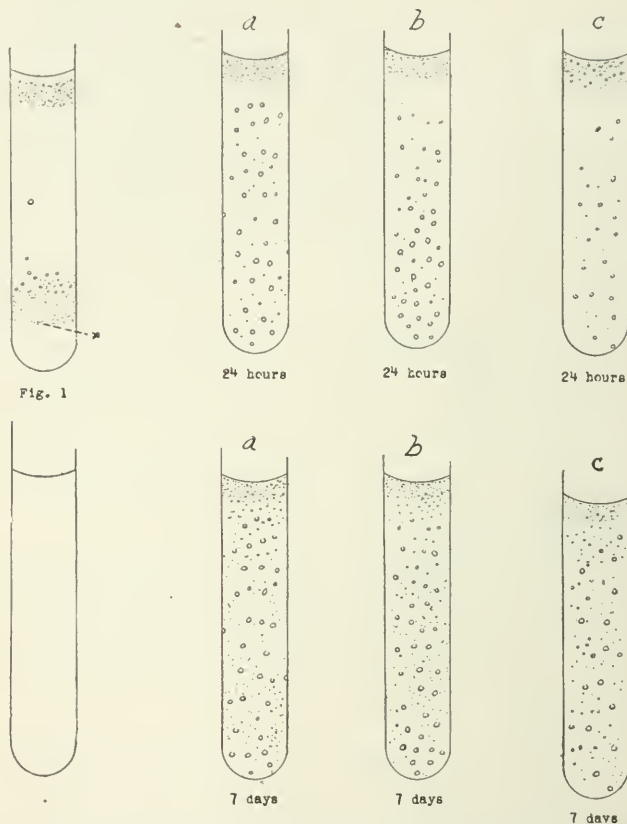


Fig. 1. A deep shake culture of the streptococcus, showing 2 widely separated zones of growth; an upper, or microaerophile zone, and a lower, or aerophobe, zone.

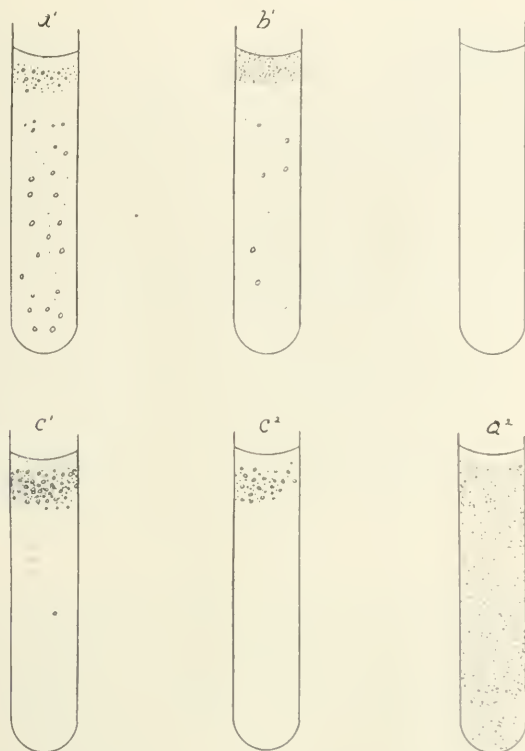
Fig. 2. A shake culture from an anaerobic subculture from X, Fig. 1.

Fig. b. A shake culture from a partial-tension subculture from X, Fig. 1.

Fig. c. A shake culture from a partial-tension subculture from the microaerophile zone (Fig. 1) at 24 hours and at 7 days. All shake cultures were made after growth for 48 hours at the corresponding oxygen-tension.

realize that our argument could have been strengthened by more extended experiments and that we should have tried to convert the microaerophile strain c' into an aerophobe. In fact, this microaerophile strain did show aerophobe variants as shown in Figs. c and c',

altho c was derived from a culture carefully cut out of the upper layer of the original deep tube, Fig. 1. Furthermore, the 7-day-old tubes, a, b, and c, showed the slow development of a large number of minute colonies not present in 24-48 hours, and these appeared at all levels.



Figs. a', b', and c'. Shake cultures to correspond with a, b, and c, but made after pure-colony subcultures were maintained at the corresponding oxygen tensions for 7 days.

Fig. a². Result of attempting to make the aerophobe strain completely aerophobic.

Fig. c². Result of attempting to make microaerophile strains completely microaerophilic.

However, we did try to get a strictly aerophobe strain from a' and a purely microaerophile one from c' as follows: Single colonies were transplanted from the slants used to seed the deep tubes a' and c'. These subcultures were designated a² and c². A² was transplanted and incubated under anaerobic conditions every day for 6 successive days. C² was kept at partial tension without transfer at the same temperature, 37 C., for the same length of time. Both of these were now tested with the results shown in Figs. a² and c².

At the same time that these culture tests, a^2 and c^2 , were made, 2 animal inoculations were performed as follows: The slant cultures, a^2 and c^2 , were suspended in sterile salt solution, sedimented, and resuspended in salt solution. Two half-grown rabbits each received 1.5 c.c. of these suspensions intravenously. The suspensions were approximately the density of a 24-hour typhoid broth culture. The rabbits were apparently well 48 hours later when they were killed. Both appeared normal on dissection except that the rabbit which had received the anaerobic strain, a^2 , showed marked congestion of the tissues about both knee joints, which were full of a bloody fluid. Anaerobic cultures from this bloody exudate remained sterile.

The cocci had been grown on Martin's pleuritic or ascitic medium for exactly a month when this inoculation was performed.

While this single experiment does not definitely support the contentions of Rosenow regarding the relationship between localization in the body and oxygen tension, it does seem to point to the greater pathogenicity or toxicity of the aerophobe strain. At any rate the technic used here, and the observation that apparently more or less uniform adaptation can be brought about by maintenance at a certain tension, opens the way for further experiments.

SUMMARY AND CONCLUSIONS

Apparently animal as well as bacterial parasites are adapted to an oxygen tension below the atmospheric. The anaerobic culture of a streptococcus, when planted in a deep tube of agar, yielded widely separated zones of colonies, microaerophile and aerophobe. A further study of these two zones showed that both strains tended to throw off variants. Maintaining the microaerophile strain at partial-oxygen tension for several days produced a fairly uniform strain. Incomplete results were obtained by daily anaerobic transfers of the aerophobe strain. Each strain was inoculated into the circulation of a rabbit a month after isolation. The results are considered inconclusive, tho there were marked hemorrhages into the knee joints of the rabbit inoculated with the aerophobe strain.

THE NUMERIC RELATIONSHIP OF INFECTION TO THE CHEMOTHERAPY OF EXPERIMENTAL TRYPANOSOMIASIS *

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In conducting experiments in the chemotherapy of experimental trypanosomiasis certain factors, as the virulence of the trypanosomes for the test animals and the time when the drug is administered, before or at varying intervals after infection, modify the results with even such a powerful trypanocide as arsenobenzol. As stated in a former communication,¹ the parasitropic effects of a drug or substance under study, and the resistance of the animal to the drug's organotropic effects, are apparently modified, to some extent at least, by an additional factor; namely, whether the animal is infected with small or large numbers of trypanosomes. The object of this paper is to record experiments bearing upon the numeric relationship of infection to the chemotherapy of experimental trypanosomiasis.

METHOD OF STUDY

In our routine chemotherapeutic tests and experiments, a series of rats is infected with a uniform number of *T. equiperdum* 24 hours before the drug is administered intravenously in dosage according to the body weight of each animal. With the number of trypanosomes inoculated by intraperitoneal injection in our tests (100,000 to 150,000 per rat), the parasites are not usually to be found in the peripheral blood until 48 hours after infection, or 24 hours after treatment. In this manner the infection is given a 24-hour start, and is heavy enough to infect the controls in every instance, yet light enough not to mask finer degrees of therapeutic effect on the part of the drug under study.

In the experiments recorded in this communication two strains of *T. equiperdum* were used. Series of rats were infected with increasing numbers of trypanosomes after the method of Kolmer,^{1,2} each two rats receiving the same number of parasites.

* Received for publication October 4, 1916.

¹ Kolmer, Jour. Infect. Dis., 1915, 17, p. 79.

² Jour. Infect. Dis., 1915, 16, p. 311.

TABLE 1
ARSENOBENZOL AND *T. EQUIPERDUM* (NEW STRAIN)

Rat	Weight, Gm.	Arsenobenzol Dose per 100 Gm.	Number of <i>T. Equiperdum</i> Injected
1.....	100	.0005	120,000,000
2.....	155	.0005	60,000,000
3.....	135	.0005	36,000,000
4.....	135	.0005	18,000,000
5.....	130	.0005	6,000,000
6.....	100	.0005	3,000,000
7.....	77	.0005	1,200,000
8.....	99	.0005	600,000
9.....	80	.0005	240,000
10.....	75	.0005	60,000
11.....	95	Control	120,000,000
12.....	73	Control	60,000,000
13.....	76	Control	36,000,000
14.....	103	Control	18,000,000
15.....	160	Control	6,000,000
16.....	70	Control	3,000,000
17.....	71	Control	1,200,000
18.....	90	Control	600,000
19.....	83	Control	240,000
20.....	69	*Control	60,000

Twenty-four hours later, half the number of infected animals received a constant dose of arsenobenzol according to the body weight of each animal, our object being to determine the influence of a constant dose of arsenobenzol upon rats infected with increasing numbers of the parasites.

The blood of each animal was examined daily for trypanosomes and the results recorded after the scheme given in Table 1.

The results were determined according to whether the animals receiving arsenobenzol remained sterile, or according to the time elapsing before trypanosomes were found in the peripheral blood and the duration of life as controlled by the untreated animals.

In numerous experiments we have found that the sterilizing dose of salvarsan (Ehrlich) and arsenobenzol (Dermatological Research Laboratories) for *T. equiperdum* according to our method, is from 0.001 to 0.002 gm. per 100 gm. of body weight (average 0.0015 gm. per 100 gm.).

Both products in dosage of 0.0005 gm. per 100 gmi. of body weight inhibit the multiplication of trypanosomes for a time, but do not usually sterilize the animal.

The results of an experiment with increasing numbers of *T. equiperdum* and 0.0005 gm. arsenobenzol per 100 gm. of rat, are shown in Table 1.

The "new strain" of *T. equiperdum* refers to a fresh strain kindly

TABLE 1—*Continued*
ARSENOBENZOL AND *T. EQUIPERDUM* (NEW STRAIN)

Results of Daily Examination of Blood from Tail														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Few	Few	+	++	++++	+++++	++++	D							
Few	+	++	++++	+++++	+++++	++++	+++++	D						
Few	Few	++++	+++++	+++++	+++++	D	++++							
Few	Few	++++	+++++	+++++	+++++	++++	+++++	D						
Few	+	++++	+++++	+++++	+++++	++++	+++++	+	+	D				
Few	+	++++	+++++	+++++	+++++	++++	+++++	+++++	D					
—	+	++	+++	+++	+++++	D								
—	Few	++	+++	+++++	+++++	++++	D							
—	—	—	—	+	+++++	+++++	+++++	+++++	D					
—	—	—	—	—	+++++	+++++	+++++	+++++	D					
Few	Few	++++	+++++	+++++	D	—	—	+	+	+++	+++++	+++++	D	
Few	+	++++	+++++	D										
Few	+	++++	+++++	D										
Few	+	++	++++	+++++	+++++	D								
Few	+	++++	+++++	D										
Few	+	++++	+++++	D										
—	Few	++++	+++++	+++++	+++++	+++++	+++++	+++++	D					
—	Few	++++	+++++	+++++	+++++	D								
—	—	Few	+	++++	+++++	+++++	D							

KEY TO TABLES

++++ = very large numbers; cannot be counted.
 +++ = large numbers that may be roughly counted.
 ++ = about 10-20 in a field.
 + = about 5-10 in a field.
 Few = 1-2 in a field, or in every other field.
 — = sterile.

sent us by Dr. Wade H. Brown, of Rockefeller Institute. The "old strain" refers to one in use in these laboratories for several years which had a marked virulence and an acquired resistance to the trypanocidal activity of salvarsan and arsenobenzol.

The results of a series of experiments using a constant dose of 0.001 gm. of arsenobenzol per 100 gm. of rat, with increasing numbers of trypanosomes, are shown in Tables 2 to 5.

The results of experiments using 0.002 gm. of arsenobenzol per 100 gm. of rat, with increasing numbers of trypanosomes, are shown in Tables 6 and 7.

SUMMARY OF RESULTS

As shown in the tables the time elapsing before the appearance of trypanosomes in the peripheral blood, or the true incubation, varies according to the number injected. This phase has been reported upon in a former paper.¹ When very large numbers are injected trypanosomes may be found in the peripheral blood within 24 hours after inoculation and are supposed to be in some instances, at least, the adult forms used in inoculation.

As shown in Table 1, the administration of 0.0005 gm. of arseno-

TABLE 2
ARSENOBENZOL AND T. EQUIPERDUM (OLD STRAIN)

Rat	Weight, Gm.	Arsenobenzol Dose per 100 Gm.	Number of T. Equiperdum Injected
1.....	84	.001	1,140,000
2.....	98	.001	912,000
3.....	82	.001	684,000
4.....	132	.001	456,000
5.....	76	.001	228,000
6.....	74	.001	114,000
7.....	93	.001	91,200
8.....	90	.001	68,400
9.....	76	Control	1,140,000
10.....	80	Control	912,000
11.....	68	Control	684,000
12.....	75	Control	456,000
13.....	75	Control	228,000
14.....	70	Control	114,000
15.....	65	Control	91,200
16.....	74	Control	68,400

TABLE 3
ARSENOBENZOL AND T. EQUIPERDUM (OLD STRAIN)

Rat	Weight, Gm.	Arsenobenzol Dose per 100 Gm.	Number of T. Equiperdum Injected
1.....	82	.001	4,840,000
2.....	77	.001	2,884,000
3.....	75	.001	1,936,000
4.....	71	.001	968,000
5.....	103	.001	764,000
6.....	75	.001	580,000
7.....	55	.001	387,200
8.....	55	.001	193,600
9.....	57	.001	96,800
10.....	73	.001	77,440
11.....	50	.001	58,000
12.....	55	.001	38,000
13.....	45	Control	4,840,000
14.....	86	Control	2,884,000
15.....	80	Control	1,936,000
16.....	65	Control	968,000
17.....	60	Control	764,000
18.....	67	Control	580,000
19.....	67	Control	387,200
20.....	53	Control	193,600
21.....	60	Control	96,800
22.....	57	Control	77,440
23.....	81	Control	58,000
24.....	62	Control	38,000

TABLE 2—Continued
ARSENOBENZOL AND T. EQUIPERDUM (OLD STRAIN)

Results of Daily Examination of Blood from Tail													
1	2	3	4	5	6	7	8	9	10	11	12	13	14
—	—	—	—	Few	+	++	+++++	+++++	D				
—	—	—	—	—	+	++	+++++	+++++	+++++	D			
D*	—	—	—	—	—	—	—	++	+++++	+++++	+++++	D	
—	—	—	—	—	—	—	—	D	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
Few	+	++	+++	+++++	+++++	D			—		—	—	—
—	—	Few	+	++	+++++	+++++	D						
—	—	—	Few	++	+++++	+++++	D						
—	—	+	++	+++++	+++++	+++++	D						
D*	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	Few	+	++	+++++	+++++	D				
—	—	—	—	—	+	++	+++++	+++++	+++++	D			

* Accidental.

TABLE 3—Continued
ARSENOBENZOL AND T. EQUIPERDUM (OLD STRAIN)

Results of Daily Examination of Blood from Tail														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Few	+++	+++	+++++	D										
—	+	++	+++++	D										
—	—	Few	++	+++++	+++++	+++++	D							
—	D*													
—	—	+	+++	+++++	D		+++	+++++	D					
—	—	—	—	+	+	++	D	+++++						
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	D						
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Few	+++	D												
Few	+	+++++	+++++	D										
Few	+	+++	+++++	D										
—	+	+++	+++++	+++++	+++++	D								
—	+	+	+++	+++++	+++++	D								
—	+	+++	+++++	+++++	D									
—	—	Few	+++	+++++	+++++	D								
—	—	+	+++	+++++	+++++	D								
—	—	—	—	+	++	+++	+++++	+++++	D					
—	—	—	+	+	+	+++	+++++	+++++	D					
—	—	—	—	Few	+	+	+++	+++++	+++++	+++++	D			
—	—	—	—	—	Few	+	++	+++++	+++++	+++++	D			

* Accidental.

TABLE 4
ARSENOBENZOL AND T. EQUIPERDUM (NEW STRAIN)

Rat	Weight, Gm.	Aresenobenzol Dose per 100 Gm.	Number of T. Equiperdum Injected			
				1	2	3
1	122	.001	40,000,000	—	—	—
2	139	.001	24,000,000	—	—	—
3	118	.001	16,000,000	—	—	—
4	117	.001	8,000,000	—	—	—
5	127	.001	4,000,000	—	—	—
6	108	.001	2,400,000	—	—	—
7	127	.001	1,600,000	—	—	—
8	80	.001	800,000	—	—	—
9	110	.001	400,000	—	—	—
10	116	.001	160,000	—	—	—
11	101	.001	80,000	—	—	—
12	138	.001	40,000	—	—	—
13	129	Control	40,000,000	++	++++	++++
14	121	Control	24,000,000	Few	Few	Few
15	90	Control	16,000,000	Few	Few	Few
16	70	Control	8,000,000	++	++++	++++
17	88	Control	4,000,000	Few	++	++
18	120	Control	2,400,000	—	++	++
19	113	Control	1,600,000	—	++	++
20	127	Control	800,000	—	—	++
21	117	Control	400,000	—	—	—
22	87	Control	160,000	—	—	—
23	107	Control	80,000	—	—	—
24	70	Control	40,000	—	—	—

TABLE 5
ARSENOBENZOL AND T. EQUIPERDUM (NEW STRAIN)

Rat	Weight, Gm	Aresenobenzol Dose per 100 Gm.	Number of T. Equiperdum Injected			
				1	2	3
1	141	.001	578,000,000	—	—	—
2	121	.001	192,600,000	—	—	—
3	124	.001	96,300,000	—	—	—
4	137	.001	38,520,000	—	—	—
5	135	.001	19,260,000	—	—	—
6	110	.001	9,630,000	—	—	—
7	158	.001	3,852,000	—	—	—
8	109	.001	1,926,000	—	—	—
9	95	.001	963,000	—	—	—
10	95	.001	385,200	—	—	—
11	116	.001	192,600	—	—	—
12	140	.001	96,300	—	—	—
13	135	Control	578,000,000	++++	D	++
14	100	Control	192,600,000	+	+	++
15	90	Control	96,300,000	++	++++	++++
16	140	Control	38,520,000	++	++++	++++
17	108	Control	19,260,000	—	+	++
18	148	Control	9,630,000	—	+	++
19	132	Control	3,852,000	—	+	++
20	140	Control	1,926,000	+	++++	++++
21	115	Control	963,000	—	+	++
22	96	Control	192,300	—	Few	+
23	80	Control	96,300	—	—	+

TABLE 6
ARSENOBENZOL AND T. EQUIPERDUM (OLD STRAIN)

Rat	Weight, Gm.	Arsenobenzol Dose per 100 Gm.	Number of T. Equiperdum Injected			
				1	2	3
1	95	.002	4,360,000	+	++++	++++
2	160	.002	2,180,000	Few	+	++++
3	110	.002	654,000	—	—	+
4	108	.002	327,000	—	—	+
5	94	.002	218,000	—	—	+
6	85	.002	192,000	—	—	—
7	108	.002	174,000	—	—	—
8	115	.002	130,000	—	—	—
9	87	.002	87,000	—	—	—
10	77	.002	21,000	—	—	—
11	94	.002	13,000	—	—	—
12	110	.002	2,000	—	—	—
13	85	Control	4,360,000	++	++++	D
14	92	Control	2,180,000	++	++++	D
15	80	Control	654,000	Few	+	+++
16	92	Control	327,000	—	Few	+
17	83	Control	218,000	—	Few	+
18	127	Control	192,000	—	—	Few
19	112	Control	174,000	—	—	Few
20	90	Control	130,000	—	—	Few
21	72	Control	87,000	—	—	Few
22	100	Control	21,000	—	—	—
23	95	Control	13,000	—	—	—
24	132	Control	2,000	—	—	—

TABLE 7
ARSENOBENZOL AND T. EQUIPERDUM (NEW STRAIN)

Rat	Weight, Gm.	Aresenobenzol Dose per 100 Gm.	Number of T. Equiperdum Injected			
				1	2	3
1	121	.002	40,000,000	—	—	—
2	118	.002	24,000,000	—	—	—
3	100	.002	16,000,000	—	—	—
4	110	.002	8,000,000	—	—	—
5	124	.002	4,000,000	—	—	—
6	60	.002	2,400,000	—	—	—
7	90	.002	1,600,000	—	—	—
8	135	.002	800,000	—	—	—
9	118	.002	400,000	—	—	—
10	79	.002	160,000	—	—	—
11	73	.002	80,000	—	—	—
12	100	.002	40,000	—	—	—
13	104	Control	40,000,000	Few	++++	++++
14	93	Control	24,000,000	Few	+	++++
15	60	Control	16,000,000	Few	+++	++++
16	98	Control	8,000,000	Few	++++	++++
17	72	Control	4,000,000	Few	+	+
18	69	Control	2,400,000	Few	+	+
19	69	Control	1,600,000	Few	++	++
20	72	Control	800,000	—	+	+
21	70	Control	400,000	—	—	+
22	73	Control	160,000	—	—	—
23	80	Control	80,000	—	—	—
24	68	Control	40,000	—	—	—

TABLE 6—Continued
ARSENOBENZOL AND T. EQUIPERDUM (OLD STRAIN)

Results of Daily Examination of Blood from Tail											
4	5	6	7	8	9	10	11	12	13	14	15
D ++++ ++ ++ ++++	D ++ ++++ ++++	++++ ++++ ++++ ++++	D D ++++	++++	++++	++++	D Few	+	++	++++	D
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	D	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
D ++ ++ + ++ Few + — —	++++ ++++ ++ ++ + Few — —	++++ ++++ ++++ ++++ ++ ++ Few —	D ++++ ++++ D ++++ ++++ ++ —	D ++++ ++++ ++++ ++ ++ —	++++ ++++ ++++ ++++ ++ ++ ++ —	D D ++++ ++++ ++++ ++ ++ —	++++ ++++ D ++	++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++	D ++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++	D ++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++	D ++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++

TABLE 7—Continued
ARSENOBENZOL AND T. EQUIPERDUM (NEW STRAIN)

Results of Daily Examination of Blood from Tail											
4	5	6	7	8	9	10	11	12	13	14	15
—	—	—	—	—	+	++	+++	++++	++++	D	++++
—	—	—	—	—	—	—	—	+	++	++++	++++
—	—	—	—	—	—	—	—	—	Few	Few	Few
—	—	—	—	—	—	—	—	—	—	Few	Few
—	—	—	—	—	—	—	—	—	—	Few	Few
—	—	—	—	—	—	—	—	—	—	Few	Few
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
D ++++ ++++ + ++ ++++ ++++ + + —	++++ ++++ + ++++ ++++ ++++ ++ ++ —	++++ ++++ ++++ ++++ ++++ ++++ ++ ++ —	D ++++ ++++ ++++ ++++ ++++ ++++ ++ ++ —	++++ ++++ ++++ ++++ ++++ ++++ ++ ++ —	++++ ++++ ++++ ++++ ++++ ++++ ++ ++ —	++++ ++++ ++++ ++++ ++++ ++++ ++ ++ —	D D D D D D D D	++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++	++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++	D ++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++	++++ ++++ ++++ ++++ ++++ ++++ ++++ ++++

benzol per 100 gm. of body weight did not influence the trypanosomiasis of those rats infected with 600,000 or more trypanosomes; with smaller numbers of trypanosomes there was a delay or inhibition of development.

In general terms the administration of 0.001 gm. of arsenobenzol per 100 gm. of body weight sterilized rats infected with about 400,000 or less of trypanosomes; when larger numbers of trypanosomes were used in inoculation, sterilization did not result but the appearance of trypanosomes in the peripheral blood was delayed as compared with the controls receiving trypanosomes alone (Tables 2, 3, 4, and 5).

The administration of 0.002 gm. of arsenobenzol per 100 gm. of body weight usually sterilized rats receiving as many as 2,000,000 to 4,000,000 trypanosomes (Table 7). With the resistant strain, which was used in this experiment shortly before being discarded for the purpose of chemotherapeutic researches, the effect of this dose was much less marked (Table 6). As previously stated, this dose of salvarsan or arsenobenzol usually sterilizes animals infected with what may be called a heavy infection, namely, 1,000,000 parasites per rat.

CONCLUSIONS

These experiments demonstrate that in the chemotherapy of trypanosomiasis there is an important relationship between the number of trypanosomes injected into the test animal and the trypanocidal activity on the part of the drug.

This relationship is particularly in evidence with respect to the amount of drug necessary to effect complete sterilization; while rats infected with 500,000 trypanosomes may be sterilized with 0.001 gm. of arsenobenzol per 100 gm. of rat, this is not the case when larger numbers of trypanosomes are injected.

The influence of numbers is less marked when the rats are very heavily infected, as with numbers over 2,000,000. In these instances, arsenobenzol or salvarsan in dose of 0.001 gm. or 0.6 gm. per 60 kilograms of body weight retards the appearance of trypanosomes in the peripheral blood, but does not sterilize. The time of appearance of the parasites in the blood is likewise not greatly influenced by variation in the number used in inoculation.

The numeric relationship of infection to the results of chemotherapeutic experiments is therefore a subject of considerable importance, and more particularly in comparative tests.

STUDIES OF THE BETA HEMOLYTIC STREPTOCOCCUS (SMITH AND BROWN)*

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CONTENTS

INTRODUCTION

THE CAUSATIVE AGENT OF THE DORCHESTER MILK-BORNE EPIDEMIC
OF SEPTIC SORE THROAT, 1915

AN EPIDEMIC OF TONSILLITIS AT THE K. BOARDING SCHOOL

THE PRESENCE OF THE BETA HEMOLYTIC STREPTOCOCCUS IN NOR-
MAL THROATS

AN EPIDEMIC DUE TO INFECTION BY CONTACT WITH CARRIERS OF
THE BETA HEMOLYTIC STREPTOCOCCUS

CARRIERS. A STUDY OF THROAT CULTURES FROM 20 INDIVIDUALS
WHO HAD HAD SEPTIC SORE THROAT WITHIN THE PAST
TWO YEARS

THE PREVALENCE OF THE BETA HEMOLYTIC STREPTOCOCCUS IN
SPORADIC TONSILLITIS

THE PREVALENCE OF THE BETA HEMOLYTIC STREPTOCOCCUS IN
SCARLET FEVER

SUMMARY

CONCLUSIONS

TECHNIC

INTRODUCTION

Septic sore throat, or epidemic tonsillitis, is an acute, severe, communicable disease prevalent in the United States. It has not been clearly separated from sporadic tonsillitis, and in some instances has been confused with scarlet fever. The physician has no definite clinical or laboratory criteria on which to base a diagnosis. If a case of tonsillitis is severe, with high fever, prostration, ulceration of, and exudate on, the tonsils, and angina, the conscientious physician properly reports the case as septic sore throat; other physicians do not report such a case unless the disease is present in the community in epidemic proportions. In the mind of the health officer septic sore throat, when epidemic, is a disease due to the use of contaminated milk. There is so much confusion about the disease that any facts which tend to clarify the problem of its etiology, and more particularly the mode of its transmission, are of scientific interest and practical importance.

* Received for publication October 10, 1916. Awarded the Royleston medical prize, 1916.

HISTORICAL REVIEW

Severe inflammations of the throat due to the use of contaminated milk have been recognized in England¹ for more than 30 years. In this country, before the year 1908 no epidemics of tonsillitis due to the drinking of contaminated milk had been reported. Following the epidemiologic studies of the Boston epidemic by Winslow,² however, at least two epidemics of septic sore throat each year have been reported as due to the use of contaminated milk or milk products.

The most extensive studies of this disease are those of the Boston epidemic by Winslow; of the Chicago epidemic in 1912 by Capps and Miller,³ and Davis⁴ and others; and of the Baltimore epidemic by Frost,⁵ and Hamburger.⁶ Streptococci from the first have been believed to be the etiologic factor. Careful study of the characteristics of the streptococci isolated has been made in only a few instances.

Rosenow⁷ in 1912 carefully studied the streptococcus from the Chicago epidemic. He described it as (1) virulent for rabbits, (2) growing abundantly in ordinary media, (3) encapsulated, (4) without long chains, and (5) with but little hemolysis. His experiments tended to show that pyogenic streptococci, when placed in unheated milk, were transformed into the type found in septic sore throat. In a publication of the same year Davis and Rosenow,⁸ describing the streptococcus from the Chicago epidemic in greater detail, differentiated the streptococcus of septic sore throat from *Streptococcus pyogenes*, or the common hemolytic streptococcus, by (1) the relatively narrow zone of hemolysis, beginning immediately around the colony, with an outer indistinct zone, (2) the presence of a capsular substance, (3) the nonfermentation of inulin, and (4) virulence for animals. They suggested that it lay midway between *Streptococcus pyogenes* and *Streptococcus mucosus*. Davis⁴ added to these characteristics the fermentation of dextrose, lactose, maltose, saccharose, and dextrin, and the nonfermentation of mannite, raffinose, and inulin.

Hamburger⁶ described the etiologic agent of the Baltimore epidemic as a short-chained streptococcus with a "halo" which in media tends to form long chains. Blood agar showed a hemolytic zone. Inulin was not fermented.

Theobald Smith and J. Howard Brown⁹ (1915), in studies of streptococci isolated from presumably milk-borne epidemics of tonsillitis in Massachusetts (1913-1914), found certain definite characteristics: (1) A definite type of hemolysis (called by them the " β type of hemolysis"), which gave a sharply defined, clear, transparent, completely hemolyzed, colorless zone from 2 to 4 mm. in diameter around the colony. Under the microscope no corpuscles were seen within the zones. The colonies were simple or biconvex, never complex. (2) Fermentation of salicin, nonfermentation of raffinose and inulin. (3) Characteristic virulence for rabbits.

In further studies of streptococci isolated from the Boston, Baltimore, and Chicago epidemics, Smith and Brown found in each instance a streptococcus

¹ Bacteriology of Milk, 1903.

² Jour. Infect. Dis., 1912, 10, p. 72.

³ Jour. Am. Med. Assn., 1912, 58, p. 1848.

⁴ Ibid., p. 1852.

⁵ Bull. 5, Hyg. Lab., Washington, D. C., p. 1889.

⁶ Jour. Am. Med. Assn., 1912, 58, p. 1109.

⁷ Jour. Infect. Dis., 1912, 11, p. 338.

⁸ Jour. Am. Med. Assn., 1912, 58, p. 773.

⁹ Jour. Med. Research, 1914, 31, p. 455.

of this same type. All the types isolated did not have identical carbohydrate reactions; one group did not ferment lactose, and one group fermented mannite, tho most of the groups did not. Smith and Brown accorded no emphasis to capsule-formation or length of chain as constant or significant.

An important point brought out by Smith and Brown was that these streptococci were, they believed, of human origin with characteristics readily differentiable from those of bovine strains. Septic sore throat, they held, is not due to a bovine strain of streptococci from a "gargety" cow. Udders of cows become infected with human streptococci, which are only mildly pathogenic for cattle, but which may remain in the udder reservoir for some time, multiplying and heavily contaminating the milk. In Outbreak B, Smith and Brown isolated from a cow a streptococcus which had characteristics similar to those of the human strains of the same epidemic.

These observations were substantiated by the studies of Krumwiede and Valentine,¹⁰ who isolated a streptococcus from the udder of a cow in a dairy from which infective milk had come, which in its characteristics was similar to the beta hemolytic type. Inflammation of the cow's udder was slight. Agglutination tests further identified the streptococci from the throats of patients with those from the udder of the cow. One dairy worker had a sore throat, but the authors were unable to isolate the beta hemolytic streptococcus from him.

The probable method of infection of the cow's udder with a human strain of streptococcus is demonstrated in the experiments of Davis and Capps.¹¹ They infected a small abrasion on the skin of the teat with streptococci from tonsillitis and septic sore throat; slight redness and tenderness resulted, and the human strains of streptococci were present in the milk at the end of 4 weeks. A like result followed injection of a culture of a human strain directly into the duct. The streptococci did not lose their characteristics during the 4 weeks in the udder of the cow.

The essential characteristics which, according to Smith and Brown, distinguish this streptococcus as a human strain have each received consideration by other investigators.

Carbohydrate Reactions.—The best recent work on the carbohydrate reactions is that by Broadhurst,¹² which includes a review of the literature together with a study of over 700 strains of streptococci. She concludes that it is impossible to differentiate human from bovine strains by means of the carbohydrate reactions.

Hemolysis.—The results of study of streptococci from the standpoint of hemolysis have been unsatisfactory because of the lack of a standard technic. *Streptococcus pyogenes* has been generally recognized as hemolytic, but without any clear definition of the type of its hemolysis. Different workers have used the blood of different animals, mixing the blood with agar prepared in various ways, and in varying amounts, and reading the hemolytic plates at varying times after incubation. Comparative results are therefore almost without value.

Ruediger,¹³ as early as 1912, suggested a differentiation of *S. pyogenes* in milk from *S. lactis* by the fact that *S. pyogenes* produces small colonies in blood agar, surrounded by a large zone of hemolysis, while *S. lactis* produces

¹⁰ Jour. Med. Research, 1915, 28, p. 231.

¹¹ Jour. Infect. Dis., 1914, 15, p. 135.

¹² Ibid., 1915, 17, p. 277.

¹³ Science, 1912, 35, p. 223.

grayish or greenish colonies, with little or no hemolysis. Puppel¹⁴ brought out the same facts. Later studies have shown that hemolytic streptococci may be found in bovine feces (Broadhurst¹²) and thence in milk. It has also been shown that all hemolytic streptococci in milk are not pathogenic for rabbits. Hemolysis alone is therefore not sufficient basis for differentiating human from bovine strains.

Virulence for Animals.—Virulence for animals as a basis for the classification of streptococci, has received less attention than carbohydrate-fermentation and hemolysis. Rosenow,⁷ in an epidemic of septic sore throat, isolated a streptococcus with characteristic cultural properties from both the milk and the patients by intravenous injection of a suspension of the suspected material into rabbits; a characteristic polyarthritis was produced in the rabbits, from which pure cultures of hemolytic streptococci were obtained. He did not think it possible to differentiate human from bovine strains of streptococci, because the streptococci were so modified in the milk as to be transformed into the types found in septic sore throat. Jackson¹⁵ also obtained a characteristic polyarthritis in the rabbits by injection of streptococci from the Chicago milk epidemic.

I have had the opportunity to repeat the work of Smith and Brown in 2 milk-borne epidemics of septic sore throat. These were studied from both epidemiologic and bacteriologic points of view. In each epidemic a strain of streptococci was isolated from the throats of the patients and from the milk, and in one case from the udder of the cow, which corresponds in all its characteristics with the type of streptococcus reported by Smith and Brown.

Since the beta hemolytic streptococcus appears to be a human strain, it must follow that milk can be contaminated only from some human source — directly by handling, or indirectly through infection of the cow's udder. This being the case, it is of importance to know the prevalence of this strain of streptococci in human throats, the discharges from which are the most widely disseminated of all human excreta. Is the beta hemolytic streptococcus present in normal throats? If so, the discharges from the mouths of all of us are a potential source of danger. Is the beta hemolytic streptococcus found in the throats of individuals after recovery from septic sore throat, and how long is the individual, in such case, a source of danger to the community? Does septic sore throat occur sporadically, or only in epidemic form? If sporadically, what proportion of ordinary tonsillitis is due to the beta hemolytic streptococcus? Finally, what relation has the beta hemolytic streptococcus to scarlet fever?

In answer to these questions I submit the results of the study of 100 cultures from normal throats, and of cultures from the throats

¹⁴ Ztschr. f. Hyg. u. Infektionskrankh., 1912, 71, p. 3.

¹⁵ Jour. Infect. Dis., 1913, 12, p. 364.

of 20 individuals who have had tonsillitis in a definitely milk-borne epidemic within the past 2 years. A contact epidemic of septic sore throat due to the beta hemolytic streptococcus also is reported because it seems to illustrate the connection between sporadic and epidemic occurrences of the disease. The presence of the beta hemolytic streptococcus in the throats of 20 patients with typical follicular tonsillitis is noted and also the results of the study of cultures from the throats of patients suffering from various types of scarlet fever.

THE CAUSATIVE AGENT OF THE DORCHESTER MILK-BORNE
EPIDEMIC OF SEPTIC SORE THROAT, 1915

On April 23, 1915, an epidemic of sore throat was reported to the Boston board of health from the suburb of Dorchester. There had been no sporadic cases reported previous to this date, nor had there been undue incidence of tonsillitis. The epidemic, which lasted for 6 or 7 days, was practically limited to Dorchester.

EPIDEMIOLOGIC STUDIES

During the week of April 23 to May 1, 295 cases of septic sore throat were reported in the Dorchester district. Of these, 57 were not typical clinically, 5 were definitely diagnosed as scarlet fever, and 6 were not found by the health officer. The remaining 227 cases were typical of septic sore throat.

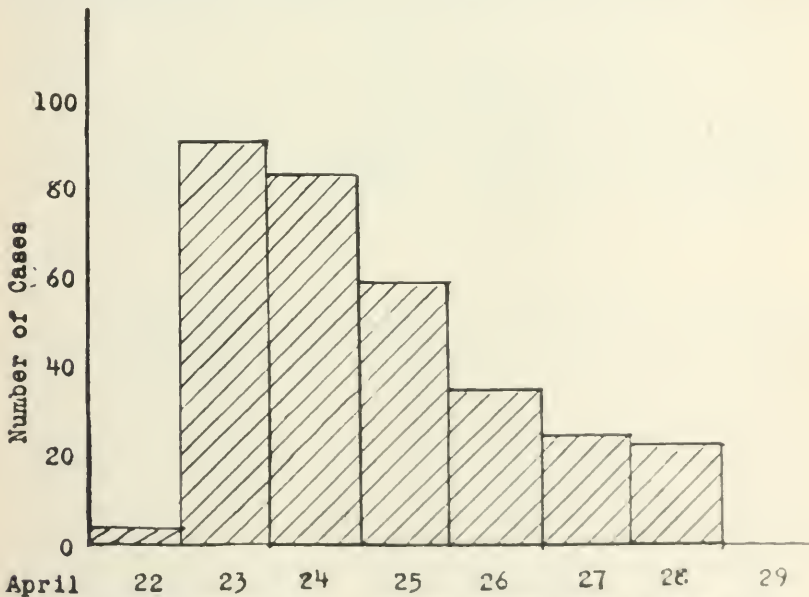


Chart 1. Case incidence of the Dorchester epidemic of septic sore throat

The curve of case incidence, shown in Chart 1, is rather typical of a milk-borne epidemic. This curve is really a record of the cases as reported to the board of health, but since most of the cases were reported on the first day of the disease it closely portrays the true case incidence of the outbreak.

There was a variation in the virulence of the disease: 50% of the patients were only slightly ill; 40% were moderately ill; and 10% were seriously ill, two deaths being directly attributable to the acute disease. Several succumbed to a resulting pneumonia or to other complications, of which endocarditis and otitis media were the most common. There were no cases of peritonitis.

An investigation of the milk supply of the 227 typical patients gave the following results:

Oak Grove milk, pasteurized.....	5 cases
Manning's milk, not pasteurized.....	12 cases
Pond's milk, not pasteurized.....	203 cases
Codman's milk, pasteurized.....	5 cases
Gushee's milk, not pasteurized.....	1 case
Moore's milk, not pasteurized.....	1 case
Garrity's milk, not pasteurized.....	1 case
McShane's milk, not pasteurized.....	1 case
Redmond's milk, not pasteurized.....	1 case

The conspicuous Pond dairy is a comparatively small one, supplying only a small proportion of the milk used in Dorchester, but almost 90% of the typical cases were on Pond's milk route. Pond supplied milk to Dorchester only, and the epidemic was limited to Dorchester. It seems most probable, therefore, that Pond's milk was the cause of the epidemic.

This dairy was the distributing station of 3 small farms (C. F. and B.), Pond owning the milk route, the wagons, the bottles, etc. There had been no illness among any of the farmers except in the case of one lad (A. B.) of 19 years, who 3 weeks previously had had sore throat and fever. No rash had been noted, but at the time of visit the health officer noted a characteristic desquamation of the hands and feet. The boy had received practically no medical attention, had been in bed but a few days, and was at the time of visit in good health. During his convalescence he had been helping with the milking. The board of health considered it probable that this boy had had scarlet fever, and had been most likely the source of the contamination of the milk.

BACTERIOLOGIC STUDIES

On April 26, throat cultures were obtained from swabs collected by Dr. Ceconi, the district health officer, from all those who were connected with the preparation of Pond's milk for the market, and from typical cases of the disease. A sample of Pond's milk, taken April 23, was also obtained for study.

The throat swabs were at once placed in 10 c.c. of normal salt solution. One small platinum loop (2 mm. in diameter) of this suspension was added to 10 c.c. of meat infusion agar containing 1 c.c. of horse blood. Plates were then poured and incubated. At the end of 24 and 48 hours the plates were studied, and colonies suggestive of the beta type of hemolysis could be isolated. In case the colonies on the plate were too numerous, duplicate plates were made with one-tenth the first amount of suspension (the normal salt suspension being kept in the ice box for just this emergency).

Table 1 gives a summary of the clinical observations and preliminary laboratory findings. The throats of all the patients, the sample of milk, and

the throats of 5 of the dairymen yielded streptococci of the beta hemolytic type. Those patients who were acutely ill gave larger numbers than those who were moderately ill. Once or twice the beta type was found in a throat which to all clinical appearances was normal.

In order to determine whether the hemolytic streptococci found in the throats of the patients and the dairymen, and in the milk from Pond's dairy, were identical, further cultural studies were undertaken. Typical colonies of

TABLE 1

CLINICAL AND PRELIMINARY LABORATORY OBSERVATIONS IN THE DORCHESTER MILK-BORNE EPIDEMIC

Source of the Strain	Clinical Condition		Beta Hemolytic Streptococci from Throat
	General	Local	
Milker on O. farm	Excellent.....	Throat apparently normal.....	None
Milker on O. farm	Excellent.....	Throat slightly congested.....	None
Worker on O. farm	Not prostrated; at work in dairy	Throat deeply congested.....	100 clear-cut colonies in almost pure growth
Worker on F. dairy	Prostration. Fever 102....	Deep injection of throat, ulceration of and exudate on tonsils	Large numbers
Worker on F. farm	Excellent.....	Throat apparently normal.....	8-10 colonies
Worker on F. farm	Excellent.....	Throat apparently normal.....	8-10 colonies
Worker on F. farm	Excellent.....	Throat apparently normal.....	None
Worker on F. farm	Excellent.....	Throat apparently normal.....	None
Milker on B. farm	No acute disease; general condition poor	Chronic inflammation of throat..	None
Worker on B. farm	No prostration, no fever....	Throat injected and slightly painful	None
Worker on B. dairy	No prostration, no fever....	Throat slightly injected, profuse nasal discharge	Almost pure growth
Worker on B. dairy	Desquamation of hands and feet	Throat apparently normal. Acute sore throat and fever 3 weeks previously	None
Patient....	Slight prostration. Fever 99-100	Throat deeply injected, no ulceration	4-6 colonies to each blood-agar plate
Patient....	Slight prostration. Fever 99-100	Throat deeply injected, no ulceration	8-10 colonies to each blood-agar plate
Patient....	Severe prostration. Fever 103-104	Ulceration of throat, exudate on tonsils	Large numbers to each plate
A patient who did not use Pond's milk	Mild prostration, slight fever	Throat injected, no ulceration....	None
Patient....	Moderate prostration. Fever 101-102	Throat deeply injected, no ulceration	Almost pure growth
Patient....	Marked prostration. Fever 102-104	Throat ulcerated, exudate and pseudomembrane on tonsils	Almost pure growth
Pond's milk (sample received from board of health).....			Large numbers

the beta hemolytic type were isolated from the plate cultures from 4 of the patients, from the dairymen who had positive cultures, and from the milk.

A fresh horse-blood-agar plate was made for each streptococcus, and read at the end of 24 hours' incubation, plates containing more than 50 colonies being disregarded. In each instance there was a clear-cut zone of hemolysis, 3 mm. in diameter, at the end of 24 hours. Under low power all the outlines of the red cells in the hemolyzed zone had disappeared.

The colonies in the depths of the medium were disc-shaped, the borders

regular and symmetrical. On meat-infusion-agar slants, the colonies were moist, grayish-white, and discrete. In meat-infusion broth the length of chain was from 8 to 10 cocci, tho from 20 to 30 were sometimes seen in one chain. When freshly isolated, the cocci had a capsular substance, variable in amount, but usually about twice the diameter of the coccus.

TABLE 2
REACTIONS OF THE STREPTOCOCCI FROM THE DORCHESTER EPIDEMIC IN STANDARD SUGAR MEDIA

No. and Source	Type of Hemolysis	Dextrose	Lactose	Maltose	Saccharose	Man- nite	Sal- icin	Raffi- nose	Inu- lin
3D C. dairy	Typical beta	2.6	2.1	2.7	2.3	1.3	2.7	1.4	1.3
4D F. dairy		2.8	2.3	2.9	2.4	1.2	2.8	1.3	1.3
5D F. dairy		2.1	2.5	2.5	2.9	1.4	3.2	1.4	1.1
11D B. dairy		2.3	2.2	2.7	2.3	1.3	2.4	1.2	1.2
14D Patient		3.0	2.3	3.0	2.1	1.2	2.5	1.3	1.3
15D Patient		3.4	2.2	2.6	2.2	1.3	2.6	1.2	1.3
17D Patient		3.6	2.2	2.5	2.8	1.3	2.3	1.3	1.2
18D Patient		2.8	2.6	2.4	2.4	1.2	2.6	1.3	1.4
19D P. dairy		3.6	2.9	3.2	2.7	1.5	2.8	1.4	1.4

A glance at Table 2 will show that the fermentative reactions of the streptococci from the throats of 4 dairymen, 4 patients, and from the milk were practically identical. The degree of acidity attained in the different carbohydrate media through fermentation was seldom above 3 (3 c.c. normal NaOH per 100 c.c. of media).

Agglutination tests with the streptococci from the throats of the dairymen and the patients, and from the milk also gave identical results (Table 3).

TABLE 3
RESULTS OF AGGLUTINATION TESTS WITH STREPTOCOCCI OF THE DORCHESTER EPIDEMIC

Source of Strain Employed	Dilutions (Serum of rabbit immunized against Streptococcus S)									Control
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2500	1:5120	
18D Patient.....	++++	++++	+++	++++	++	++	+	±	—	—
4D F. dairy.....	e	e	e	++++	++++	++++	+	±	—	—
Pond's milk.....	++++	+++	+++	++++	++	++	+	±	—	—
K13 Boarding school.....	+++	++	++	+	+	±	—	—	—	—
S & B (Smith and Brown)	±	±	+++	+++	+	±	—	—	—	—
48N Normal throat.....	+++	+++	+++	+++	+	±	—	—	—	—
4NY N. Y. epidemic.....	±	—	—	—	—	—	—	—	—	—
10T Sporadic cases of ton-	—	—	—	—	—	—	—	—	—	—
11T) silitis (Table 15)	—	—	—	—	—	—	—	—	—	—

The first three organisms were from the Dorchester epidemic. 48N was a beta hemolytic streptococcus from a normal throat (Table 10). 4NY was from a milk-borne epidemic of tonsillitis in New York state. All these strains were of the beta hemolytic type and all fermented the same carbohydrates.

When 0.5 c.c. of a 24-hour broth culture of the strain from any of the cases was injected intraperitoneally into a mouse, death ensued in from 24 to 48 hours. The injection of 1 c.c. of a 24-hour broth culture intravenously into a rabbit, caused high fever with marked loss of weight, followed as a rule by severe polyarthritis, continued high fever, and continued loss of weight. The temperature returned to normal in from 2 to 3 months. In some cases there was permanent disability, with fixation of the joint.

In summary: The beta hemolytic streptococcus from the throats of patients ill with septic sore throat was identical with that from the throats of the dairymen and from Pond's milk, which had been used by almost 90% of those who were ill. This streptococcus resembled

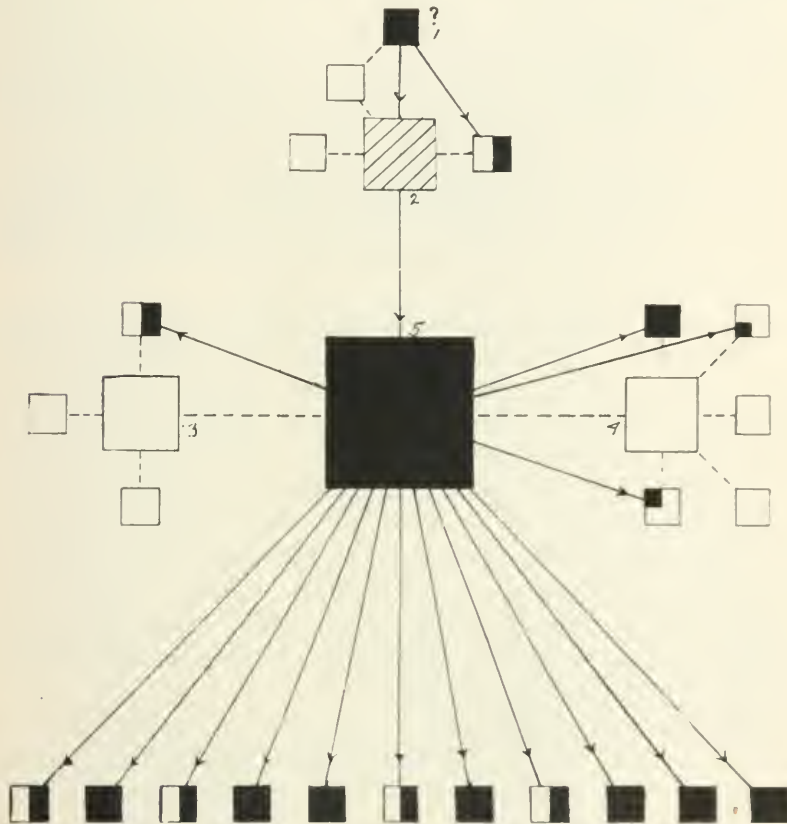


Chart 2. The probable course of the infection in the Dorchester milk-borne epidemic. (1) A. B., a supposed case of scarlet fever. (2) B. farm. (3) C. Farm. (4) F. farm. (5) Pond's distributing station. (6) Cases of septic sore throat. The black squares represent positive cultures of the beta hemolytic streptococcus. The arrows indicate the probable course of the infection.

in all its characteristics the group of streptococci isolated by Smith and Brown in their studies on milk-borne epidemics of septic sore throat.

The puzzling fact of the epidemiologic data was that the beta hemolytic streptococcus should be present in the throats of so many of the dairymen. Thus, one of the milkers of the F dairy, who was ill with septic sore throat, had large numbers of the beta hemolytic

streptococcus in his throat, but two milkers of the same dairy who had normal or but slightly affected throats, also had a few of the beta hemolytic streptococcus in their throats. At least one worker on each farm gave a positive culture of this streptococcus.

The probable explanation is that the workers of the F. farm and of the C. farm had been infected in the same way as the patients; namely, through the milk from the B. farm. The probable course of the infecting organism is shown in Chart 2. A.B., convalescing from scarlet fever (?), infected one of the cows on his father's farm with a beta hemolytic streptococcus. This cow contaminated the milk which was sent to Pond, to be further mixed with the F. and C. milk, and delivered. Thus the bottled milk became contaminated. Bottles returned to C. and F. by Pond caused infection of the milkers on the C. and F. dairies.

There are 3 weak points in these epidemiologic studies as shown in the diagram: (1) A sample of milk was not obtained from each cow of the B. farm, or of the B. milk. (2) A positive culture was not obtained from A.B., the supposed source of infection. (3) We have no proof that one of the other farms did not contaminate Pond's milk. We feel sure that E.F. of the F. farm and T.B. of the C. farm were not the source of the infection, since they developed sore throat at the same time that the other patients did. We have no knowledge as to the date of infection of G.L. or A.L. of the F. dairy. The epidemiologic evidence is therefore not conclusive. We are sure only that the epidemic was due to the beta hemolytic streptococcus, and that the source of infection was Pond's milk.

Additional facts of interest are that the same streptococcus may cause severe, moderate, or mild tonsillitis, and that the beta hemolytic streptococcus may be found in throats that are clinically normal, as in the cases of G.L. and A.L.

AN EPIDEMIC OF TONSILLITIS AT THE K. BOARDING SCHOOL

Of most importance in the study of an epidemic is ability to control, within a small compass, all possible factors pertaining to it. The epidemic of tonsillitis at the K. boarding school is for this reason of special interest. The epidemic was traced to infected milk that had been insufficiently pasteurized. The organism isolated from the throats of the patients was isolated also from the udder of one of the farm cows. It was identical with the one described by Smith and Brown as

a human strain of streptococcus. The only missing factor in the epidemiology of the outbreak was the individual who had infected the cow.

EPIDEMIOLOGIC STUDIES

There were about 160 boys in this school, living under ideal conditions for the preservation of health. There had been no sickness in the school, with the exception of one boy who had had tonsillitis 14 days before. He had been isolated at once, kept in the hospital, with his own dishes, food, attendants, etc. This case is shown in the incidence chart and included in the studies because he was considered by some to be the source of the infection.

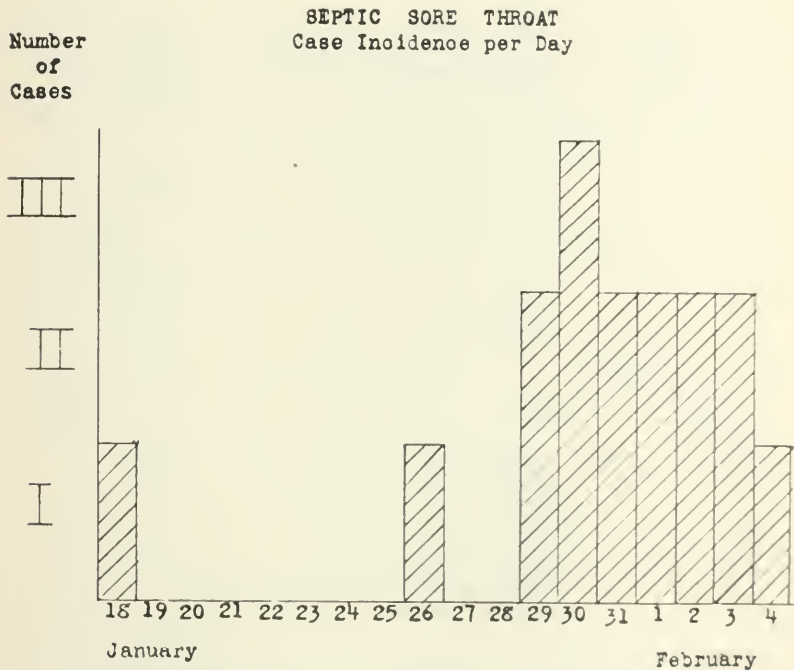


Chart 3. Case incidence of the epidemic at the K. boarding school.

There were 17 cases in the epidemic (Chart 3). A summary of each case is given in Table 4 with the clinical history and the preliminary laboratory findings.

Beta hemolytic streptococci were isolated from the throats of 15 of the boys. Four of these boys had severe septic sore throat with high fever, marked prostration, ulceration of the tonsils, pseudomembrane on the tonsils, and angina of the throat. Their convalescence was slow. Six were moderately ill with what would have been classed as ordinary tonsillitis. Four had slight sore throat with no ulceration of the tonsils and little or no fever. One had slight sore throat, but later developed severe otitis media.

All factors that could play a part in the spread of the outbreak were con-

TABLE 4
SUMMARY OF THE CLINICAL DATA, AND PRELIMINARY LABORATORY FINDINGS IN THE EPIDEMIC
AT THE K. BOARDING SCHOOL

Patient	General Condition	Local Condition	Day of Disease	Total Colonies per Plate	Hemolytic Colonies	Type of Hemolysis	Diameter of Hemolytic Zone, mm.
E1	Temp. 100-102 for 5-6 days. Moderate prostration	Ulceration of the tonsils. Moderate edema of the neck	13	150-200	75-100	Alpha type	1
K2	Temp. 101-102. Marked prostration	Marked ulceration with exudate	7	200-250	Almost pure growth	Beta	3
K3	Temp. 104. Severe prostration	Severe sore throat. Ulceration on tonsils with membrane. Moderate edema of neck	2	150-200	Almost pure growth	Beta	3
K4	Temp. 100. Moderate prostration	Throat moderately sore. No edema of the neck. No ulceration of tonsils	1	200-250	Almost pure growth	Beta	3
K5	Temp. 99.5. Slight prostration. Recovery in 2-3 days	Slight sore throat. No ulceration of the tonsils	1	150-175	20-30	No beta	1.5-2
K6	Temp. 100. Moderate prostration	Tonsillar crypts filled with exudate. No ulceration. No edema of the neck	4	100-125	Almost pure growth	Beta	3
K7	No fever. No prostration. Slight nasopharyngitis for past 10 days	Throat somewhat sore for past 3-4 days. Throat moderately injected	4?	250-300	Large numbers	All alpha type. No beta	1-2
K8	Temp. 101.5. Moderate prostration	Ulceration and slight exudate on tonsils. Deep injection of whole throat	2	150-200	Almost pure growth	Beta	3
K9	Temp. 101. Moderate prostration	Moderately sore throat. No ulceration of tonsils. No edema of the neck	6	75-100	50-60	Beta	3

sidered. The probability of contact infection seemed unlikely, because each boy was isolated as soon as he developed sore throat or fever. The only possible contact cases were K1 and K2. K1 was not considered a likely cause of the outbreak, because he had contracted the disease during the Christmas holidays, was ill on the day of his return to school, had been completely isolated from the first, had recovered from his tonsillitis, but had developed myocarditis, and was in his third week of isolation when the epidemic began. Furthermore, cultural studies of his throat did not show the beta type of hemolytic streptococcus.

The epidemic began with Case K2. The boy in this case was isolated at once. That he had got his infection from the same source as the other boys seemed probable, inasmuch as he had not been away from the school, nor exposed to sources of infection other than those to which the whole school had been exposed.

The milk, which was delivered in bulk from a farm nearby, had been

TABLE 4

SUMMARY OF THE CLINICAL DATA, AND PRELIMINARY LABORATORY FINDINGS IN THE EPIDEMIC AT THE K. BOARDING SCHOOL—*Continued*

Patient	General Condition	Local Condition	Day of Disease	Total Colonies per Plate	Hemolytic Colonies	Type of Hemolysis	Diameter of Hemolytic Zone, mm.
K10	Temp. 99.4. No prostration. Recovery in 4-5 days	Slightly sore throat. No ulceration of tonsils	5	75-80	10-15	Beta	5
K11	Temp. 99. No prostration	Throat slightly sore. Deep injection but no ulceration. Normal in 3 days	6	150-200	Almost pure growth	Beta	3
K12	No fever or prostration until complications	Slightly sore throat. Used a nasal douche, and 2 days later developed severe otitis media	5	Throat 150-200 Ear 200-250	Throat 10-15 Ear, almost pure growth 50-60	Throat, beta Ear, beta	3 3
K13	Temp. 102.4. Moderate prostration. Well in 3-4 days	Throat moderately sore. Moderate injection with exudate. No ulceration	5	100-150		Beta	5
K14	Temp. 100.4. Moderate prostration. Recovery in 6-7 days	Throat moderately sore. Deep injection, but no ulceration	3	200-250	Almost pure growth	Beta	3
K15	No fever. No prostration	Throat slightly sore, with slight injection, but no exudate and no ulceration	4	150-200	Almost pure growth	Beta	3
K16	Temp. 99.3. No prostration	Throat slightly sore, with slight injection, but no exudate and no ulceration	3	150-200	10-15	Various types No beta	
K17	No fever. No prostration	Throat slightly sore. Moderate injection. No ulceration	2	75-100	7-8	Beta	7
K18	Temp. 99.8. No marked degree of prostration	Throat deeply injected. No exudate, or ulceration	4	100-125	4-5	Beta	2

inadequately pasteurized in the school kitchen. Five-gallon cans of cold milk were placed in a vat of water, and the water heated to 170 F. for 20 minutes; the contents of the cans at the end of 20 minutes, as shown by subsequent tests, had been raised only to incubation temperature. The dairy was in an unsanitary condition, and 4 of the 50 cows in the dairy were found to have mastitis:

Cow 1.—General condition excellent. Left hind quarter of the udder slightly tender, with slight inflammation, no pus found in the milk. One small platinum loop (2 mm.) of milk from this udder added to 10 c.c. of blood agar and plated at the end of 48 hours showed in each plate from 8 to 10 colonies of hemolytic streptococci of the beta type (Table 5).

Cow 2.—Had been drying up for 2 or 3 weeks. Milk not used for several days. Udder inflamed, and milk contained large amounts of pus. No hemolytic streptococci on blood-agar plates.

Cow 3.—"Garget" 1 week, during which milk had not been used. Left hind quarter of the udder inflamed, but no pus in the milk. No streptococci of the beta type in cultures from this sample.

Cow 4.—Condemned as tuberculous 1 week before, but milk was still being used. Definite tuberculosis of left hind quarter of the udder. One small

TABLE 5

LABORATORY STUDIES OF THE HEMOLYTIC STREPTOCOCCI OF THE K. BOARDING SCHOOL EPIDEMIC

No.	Type of Hemolysis	Virulence for Animals (24-hr. broth culture)	Length of Chain (cocci)	Dextrose	Lactose	Maltose	Saccharose	Mannite	Saline	Raffinose	Inulin
K8	Beta	0.5 c.c. killed mouse in 36 hours	30-40	2.9	2.8	2.4	2.5	1.3	2.6	1.0	1.0
K7	Not a beta type. Zones 1-2 mm.	0.5 c.c. did not kill mouse	15-20, sometimes longer	2.6	3.7	3.8	3.3	1.4	1.3	1.2	1.4
Cow 1	Beta	0.5 c.c. killed mouse in 30 hours	20-30, sometimes longer	2.8	2.9	2.8	2.7	1.4	2.9	1.3	1.4
Cow 4	Not beta type. Zone 2-2.5 mm.	0.5 c.c. killed mouse in 24 hours	4-8-12	5.9	4.6	4.5	5.1	1.3	1.3	1.4	1.3
K10	Beta	0.5 c.c. killed mouse in 48 hours	30-40	3.3	3.8	3.0	2.7	1.2	2.7	1.3	1.2
K12, ear culture	Beta	0.5 c.c. killed mouse in 36 hours	20-30, some long and inter-lacing	2.9	2.3	2.7	3.3	1.0	2.8	1.1	1.0
K13	Beta	0.5 c.c. did not kill mouse	Long and inter-lacing	3.0	3.1	3.0	2.6	1.0	3.3	0.9	1.1
K14	Beta	0.5 c.c. killed mouse in 36 hours	40-50 or more	2.4	2.1	2.8	2.6	1.0	3.0	1.0	1.3
K3	Beta	0.5 c.c. killed mouse in 20 hours	30-40 or longer	3.0	2.9	2.9	2.8	1.0	3.1	1.3	1.2
K3a, 2nd isolation	Beta	0.0 c.c. killed mouse in 48 hours	30-40	2.9	2.7	3.0	2.8	1.2	2.7	1.3	1.2

loopful of the milk added to 10 c.c. of blood agar, at the end of 24-48 hours, showed on each plate from 7 to 8 colonies of hemolytic streptococci, not, however, of the beta type. The diameter of the hemolyzed zones was from 1.5 to 2 mm., but hemolysis was not complete. Reports of this study are given in Table 5.

A summary of the epidemiologic factors compelled us to conclude that the source of the epidemic was the milk, because: (1) Boys of all ages, from all the dormitories, and all the class rooms, were infected. (2) Food was the only factor common to them all. (3) The incidence curve suggested a milk epidemic. (4) The milk had not been pasteurized. (5) Hemolytic streptococci were found in the udders of 2 of the cows on the farm supplying the milk.

BACTERIOLOGIC STUDIES

Studies were now undertaken to identify the streptococci found in the throats of the boys, and to show any relation that might exist between these streptococci and those found in the udders of the diseased cows.

A number of colonies of typical beta hemolytic streptococci were isolated in pure culture from severe, moderate, and mild types of the disease; from the aural discharge of the case with mild sore throat and severe otitis media; and from the milk of the diseased cows. The characteristics noted were: type of hemolysis, appearance on meat infusion agar, length of chain in a 24-hour meat-infusion-broth culture, reactions on the standard carbohydrate media, and virulence for animals. The summary of these results in Table 5 indicates that the microorganism from the throats of the patients and that from the udder of Cow 1 are of the same strain.

The colonies on meat infusion agar in each instance were grayish-white, moist, and raised, growing readily on all ordinary infusion agar without the addition of ascitic fluid. On blood-agar plates there was a clear-cut zone of hemolysis from 2.5 to 3 mm. in diameter at the end of 24 hours. The length of chain in meat infusion broth was from 20 to 30 cocci, often shorter than 20, but seldom longer. There were a few bizarre forms in meat infusion broth (24-hour growth). Capsular substance about twice the diameter of the coccus was seen in freshly isolated strains.

Dextrose, lactose, maltose, saccharose, and salicin were fermented. Mannite, raffinose, and inulin were not. The degree of acidity in the sugar tubes at the end of 7 days was seldom carried beyond 3.

Agglutination tests furnished further evidence that the hemolytic streptococci isolated from the throats of the boys were of the same strain as those isolated from the udder of Cow 1 (Table 6).

The strains isolated from the throats of the boys and from the cow had about the same degree of pathogenicity for animals. Mice were killed in from 36 to 48 hours with the injection of 0.5 c.c. of a 24-hour broth culture into the peritoneal cavity. Rabbits injected with 1 c.c. of a 24-hour broth culture intravenously developed high fever, and severe polyarthritis, the arthritis lasting for several weeks and resulting in some instances in permanent disability.

The last link in the chain would have been the discovery of the source of infection of the cow. Cultures from the throats of the men who were connected with the production of the milk gave a hemolytic streptococcus in only one instance, Culture K7, Table 4. This man had had a slightly sore throat, but he had not been prostrated or feverish. This hemolytic streptococcus proved not to be of the same strain as those isolated from the throats of the boys or from the udder of the cow (Table 5). We are therefore unable to show the probable source of infection of the cow.

Throat cultures from 5 boys after their temperature had returned to normal and they were up and about the hospital ward, or returned to their dormitory, yielded hemolytic streptococci of the same cultural

TABLE 6

RESULTS OF AGGLUTINATION TESTS WITH STREPTOCOCCI FROM THE MILK-BORNE EPIDEMIC OF SEPTIC SORE THROAT AT THE K. BOARDING SCHOOL

Source of Strain	Dilution of Serum									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Control
Using rabbit serum immunized against the streptococcus from Cow 1										
Cow 1.....	+++	+++	+++	+++	++	+	+	±	—	—
Human (4)....	+++	+++	+++	++	++	++	++	+	—	—
Human (7)....	+++	+++	+++	+++	++	++	±	—	—	—
Human (13)....	+++	+++	+++	++	+	±	±	±	—	—
Using rabbit serum immunized against the streptococcus from Cow 4										
Cow 4.....	+++	+++	+++	++	++	++	++	+	±	—
Cow 1.....	++	+	±	—	—	—	—	—	—	—
Human (7)....	++	+	±	—	—	—	—	—	—	—
Human (4)....	+	+	±	—	—	—	—	—	—	—
Human (13)....	++	+	+	—	—	—	—	—	—	—
Using rabbit serum immunized against the streptococcus from Human Strain 4										
Cow 1.....	+++	+++	+++	++	+	+	+	+	±	—
Human (4)....	+++	+++	+++	++	++	++	++	++	±	—
Human (7)....	++	++	++	++	++	+	±	±	—	—
Human (13)....	++	++	++	++	+	+	±	±	—	—
Blood from a case of fatal scarlet fever..	±	—	—	—	—	—	—	—	—	—

TABLE 7

PERSISTENCE OF THE BETA HEMOLYTIC STREPTOCOCCUS IN THE THROATS OF PATIENTS CONVALESCENT FROM SEPTIC SORE THROAT

Patient	Present General Condition	Present Local Condition	Degree of Severity of Disease	Day of Disease	Total Number of Colonies	Number of Hemolytic Colonies	Type of Hemolysis	Diameter of Zone, mm.
K2	Temp. normal. Returned to dormitory	Throat normal	Severe	12th	200-250	3-4	Beta	3
K3	Still in bed. Moderate prostration. No fever	Glands of neck large and tender. Throat still slightly injected	Severe	8th	150-200	Almost pure growth	Beta	3
K4	Well. Discharged to dormitory 2 days ago	Slight injection of throat. Glands of neck still tender	Moderate	7th	150-175	40-45	Beta	3
K6	Well. Discharged to dormitory 3 days ago	No injection of throat	Moderate	10th	200-250	10-12	Beta	3
K8	Headache and prostration persist. No fever	Deep injection of throat. Tonsils large and ragged	Moderate	8th	200-250	Almost pure growth	Beta	3

characteristics and of the same virulence for animals as the original strains (Table 7). Inasmuch as these streptococci were present in the throats 10 days after acute symptoms had subsided, it seems probable that, in some instances at least, the virulent beta hemolytic streptococcus may remain in the throat of an individual after recovery from the disease, for weeks or even months.

These facts suggest also that the disease is not readily spread by contact, else more boys would have developed the disease from contact with the boys who returned to the dormitories carrying the infecting organism in their throats.

In summary: The same strain of the beta hemolytic streptococcus was isolated from the severe, moderate, and mild cases of septic sore throat at the K. boarding school. That is, "septic sore throat" may occur as a mild transient disease.

This strain was identical with the strain of beta hemolytic streptococci isolated from the udder of Cow 1 on the farm supplying the school with milk.

These strains of streptococci belong to the same group of streptococci that Smith and Brown isolated in their studies of epidemics of septic sore throat.

THE PRESENCE OF THE BETA HEMOLYTIC STREPTOCOCCUS IN NORMAL THROATS

Studies of the cultures of the epidemic of septic sore throat at Dorchester and the K. boarding school indicated that the beta hemolytic streptococcus may sometimes be found in throats that are clinically normal. This fact suggested a study of the occurrence of beta hemolytic streptococci in the throats of normal individuals, and in the throats of individuals who had had septic sore throat within 2 years; that is, as carriers.

METHOD OF STUDY

Cultures were taken from the throats of 100 normal individuals in the population at large, and studied on blood-agar plates by a technic similar to that used in the previous studies. Swabs were taken of the tonsils and nasopharynx and placed at once in 10 c.c. of normal salt solution. One or two large platinum loopfuls (4 mm.) of the suspension, instead of one small loopful as in the previous studies, were transferred to blood agar, the blood-agar plates being made with 10 c.c. of meat infusion agar, + .8 to + 1, with 1 c.c. of defibrinated horse blood added. The blood-agar plates were incubated at 37.5 C. and read at the end of 24 and 48 hours.

Colonies on these plates which were suggestive of the beta type were isolated in broth, and a pure culture studied on the blood-agar plate. If the strepto-

coccus cultures thus obtained in pure growth resembled in any way the beta type of hemolytic streptococcus, they were studied further as to carbohydrate reactions, virulence for animals, and other characteristics (see Tables 9 and 10).

One hundred average throats, rather than 100 normal throats, really formed the basis of this study; many of the individuals had enlarged tonsils, slight injection of the nasopharynx, or a past history of some throat trouble.

The clinical data considered were: age, tendency to sore throat, tendency to attacks of tonsillitis, date of last attack of tonsillitis, history of scarlet fever, history of septic sore throat, and present appearance of the throat. The preliminary laboratory data recorded were: the estimation of the total number of colonies on the blood-agar plate, the estimation of the total number of hemolytic colonies on each blood-agar plate, and the estimation of the number of colonies on each blood-agar plate which were suggestive of the beta type of hemolysis. A summary of the findings is presented in Table 8.

TABLE 8
HEMOLYTIC STREPTOCOCCI SUGGESTIVE OF THE BETA TYPE IN NORMAL THROATS

Summary of 100 Cases	Adults	Children Under 13 Years
Total number of cases.....	66	34
Number subject to sore throat	15	10
Number subject to tonsillitis, one attack every year or oftener	11	10
Number having had nasopharyngitis within 1 or 2 months..	15	5
Number having had tonsillitis within 2 years.....	20	13
Number having had scarlet fever	9	2
Number having had septic sore throat.....	1	1
Number showing hemolytic streptococci present.....	(2 yr. ago.) 32	(6 mo. ago) 18
Number of those who had had tonsillitis within 2 years, who showed hemolytic streptococci present in throats...	7	5
Number of those not subject to tonsillitis who showed hemolytic streptococci present in throats.....	13	11
Number of those who had had nasopharyngitis (colds) within 2 months, who showed hemolytic streptococci present in throats.....	8	2

An analysis of the cultural studies (Tables 9 and 10) shows only 1 streptococcus, No. 48, to be of the beta hemolytic type with all the typical characteristics of the Smith classification; that is, beta type of hemolysis, characteristic carbohydrate reactions, and virulence for animals. This organism came from a young man who had recently returned from an ocean cruise, and was in a hospital with a severe subacute nephritis. There was no history of scarlet fever nor of tonsillitis, and his throat appeared normal. There were two or three typical beta hemolytic colonies on each primary blood-agar plate.

Cultures from Cases 31 and 35 are interesting, having typical hemolysis and characteristic carbohydrate reactions, but no virulence for animals. Streptococci from Cases 80, 89, and 96 did not ferment lactose, being similar in this respect to the type isolated by Smith and Brown in their Outbreak A.

In summary: Hemolytic streptococci were found in about 50% of normal throats in sufficient numbers to be detected on blood-agar plates made from throat swabs.

Beta hemolytic streptococci of the Smith type were found in 1 of 100 normal throats studied.

TABLE 9

RESULTS OF STUDIES OF AVERAGE THROATS THAT HARBORED STREPTOCOCCI SUGGESTIVE OF BETA HEMOLYSIS

No.	Age	Clinical History	Appearance of Throat	History of Scarlet Fever	Hemolysis		
					Preliminary Plate		Isolation Plate
					Total Colonies	Colonies Suggestive of Beta Hemolysis	
N31	21	Tonsillitis once a year. Last attack 1½ years before	Tonsils large and ragged	No	200	6	Pure culture, typical beta type; zone 3 mm. wide
N35	29	Working in laboratory with the beta type. Self-infected 3 months before	Apparently normal	No	100-125	10-11	Typical beta type; zone 2.5 mm. wide
N48	18	Ill with subacute nephritis. Never had had tonsillitis	Apparently normal	No	150-200	2-3	Typical beta type; zone 3.5 mm. wide
N59	45	Severe tonsillitis 14 years before. Slight sore throat 1 week before	Slight pharyngitis	No	100-150	1-2	Suggestive of beta type; zone 3.5 to 4 mm. wide; colonies very small
N80	10	Severe tonsillitis 6 months before. Not well since. Came to hospital for tonsillectomy	Tonsils large and ragged. Throat injected	No	200-250	11	Not typical of beta type; incomplete hemolysis just at colony border
N83	14	Subject to tonsillitis. Had septic sore throat with 4 others of her family 8 months before	Apparently normal	No	150-175	4-5	Zone is clear-cut but narrow; 2 mm. wide
N89	12	Not subject to tonsillitis	Apparently normal	No	75-80	4-5	Suggestive of beta type; incomplete hemolysis near colony
N95	8	Subject to tonsillitis. Last attack 2 years before	Tonsils not remarkable	No	100-150	1-2	Zone 3.5-4 mm. wide; slightly granular zone near colony
N96	3	Not subject to tonsillitis	Apparently normal	No	150-175	3-4	Incomplete hemolysis near colony
N97	10	Subject to tonsillitis. Last attack 6 weeks before. Tonsils removed 4 weeks before	Throat injected	No	150-175	4-5	Zone 2 mm. wide; not typical; incomplete hemolysis

These studies have shown no direct relation between the length of chain of a streptococcus and its virulence for animals. This fact is mentioned because length of chain in a milk streptococcus is used by some municipal and state laboratories as an index to pathogenicity.

TABLE 10
CULTURAL CHARACTERISTICS OF STREPTOCOCCI SUGGESTIVE OF BETA TYPE ISOLATED FROM
100 AVERAGE THROATS

No.	Beta Type of Hemolysis	Virulence for Animals (24-hr. broth culture)	Length of Chain (cocci)	Sugar Reactions							
				Dex-trose	Lac-tose	Mal-tose	Saccha-rose	Man-nite	Salicin	Raffi-nose	Inulin
N31	Typical	0.5 c.c. did not kill mouse	8-12-16, sometimes longer	4.0	3.6	3.5	3.5	1.2	3.8	1.2	1.2
N35	Typical	0.5 c.c. did not kill mouse	8-12, never more	4.8	4.0	4.3	4.0	1.3	3.8	1.4	1.3
N48*	Typical	0.5 c.c. killed mouse in 24 hours	Interlacing masses of chains	3.5	2.3	3.8	2.8	1.1	2.3	1.2	1.3
N59	Suggestive	0.5 c.c. did not kill mouse	8-12-16, some 30-40	3.4	3.5	3.2	3.6	0.9	0.9	1.0	1.0
N80	Suggestive	0.5 c.c. did not kill mouse	4-8-12, seldom longer	5.4	1.0	4.8	5.4	1.1	4.5	1.1	5.2
N83	Not typical; zone clear-cut, but narrow	0.5 c.c. did not kill mouse	Long interlacing masses of chains	2.8	2.7	3.0	3.3	1.4	2.5	1.3	1.3
N89	Suggestive but not typical	0.5 c.c. killed mouse in 48 hours	4-8-12, some 20-30	2.4	1.3	2.1	2.4	1.2	3.0	1.2	1.2
N95	Suggestive but not typical	0.5 c.c. killed mouse in 7 days	4-8-12, some 20-30	3.0	2.5	2.8	2.5	1.0	3.4	1.3	1.4
N96	Suggestive but not typical	0.5 c.c. did not kill mouse	8-12, some longer	3.0	1.5	2.8	2.7	1.4	2.5	1.3	1.4
N97	Suggestive but not typical	0.5 c.c. killed mouse in 8 days (pericarditis)	4-8-12, many bizarre forms	5.6	5.8	4.4	5.6	1.4	4.1	4.7	4.5

* 0.5 c.c. of a 24-hour broth culture injected intravenously into a rabbit caused high fever and death in 6 days.

AN EPIDEMIC DUE TO INFECTION BY CONTACT WITH CARRIERS OF THE BETA HEMOLYTIC STREPTOCOCCUS

The study of the epidemic at the K. boarding school suggested that the beta hemolytic streptococcus might remain in the throat of an individual long after recovery from the disease, and the history of patients

who have had septic sore throat tends to substantiate this belief, since in many instances there is chronic inflammation of the throat. The danger, if any, of such persons to the community, tho it has been assumed, has not been studied.

EPIDEMIOLOGIC STUDIES AT THE B. HOSPITAL

In January, 1915, there was an epidemic of septic sore throat at the B. hospital, many of the hospital employees being ill. Some of the cases were of the severe type, complicated in 2 cases by joint-involvement; some were

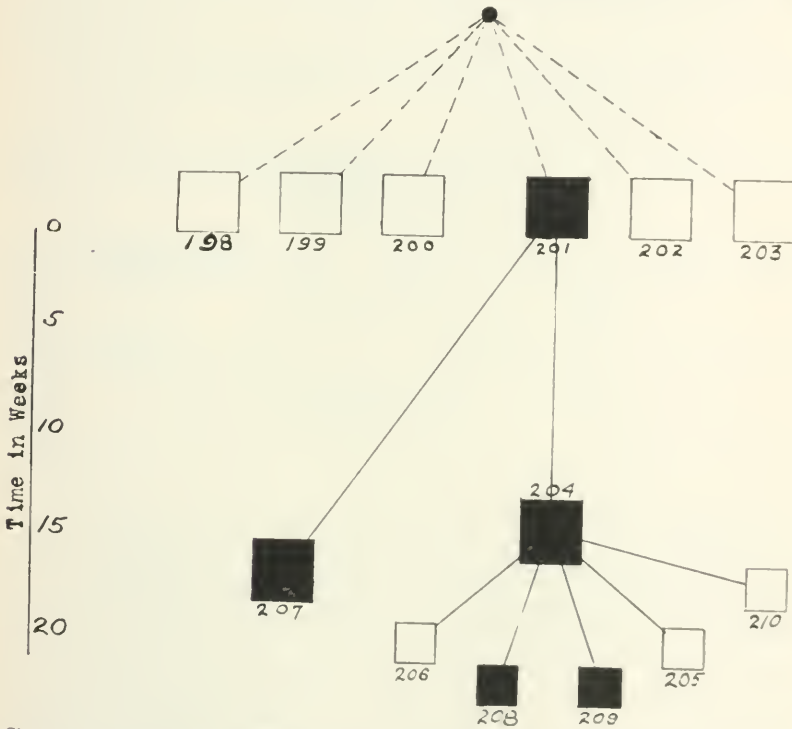


Chart 4. The probable course of a contact epidemic of septic sore throat at the B. hospital. The squares represent cultures taken. Black squares indicate positive cultures of beta hemolytic streptococci. Large squares denote employees of the hospital.

The squares numbered 198 to 203 represent cases of the milk-borne epidemic in January. Case 201 became a carrier and infected 204 and also probably 207. Case 204 probably infected the patients, since she as night nurse came in contact with all of them and had more to do with the preparation of their food than had the day nurses.

of moderate severity, and some were mild; in all the recovery was slow. The hospital authorities assumed the epidemic to be milk-borne and changed the supply of raw milk which had been used to pasteurized milk. No further cases developed.

In April, 1915, I was asked to investigate conditions at the hospital. Of every 10 patients admitted to the hospital 1 or 2 would develop tonsillitis on the 3rd or 4th day after admission. Some of the nurses also had the disease.

TABLE 11

CLINICAL CONDITION AND PRELIMINARY LABORATORY FINDINGS IN CONNECTION WITH POSITIVE CULTURES FROM THE B. HOSPITAL EPIDEMIC

No.	Source	Date of Disease	Clinical Condition		History of Tonsillitis	Type of Hemolysis	Hemolytic Colonies to Each Plate
			General	Local			
201*	Day nurse	3 months before	Had had a severe infection. Now in excellent health	Throat somewhat sore at times since the epidemic. Tonsils slightly injected	Never subject to sore throat until epidemic	Typical beta	10-15
204	Night nurse	3 weeks before	Felt tired and run down since attack. Had had a septic sore throat	Throat injected. Tonsils large and ragged	Never subject to tonsillitis until this attack	Typical beta	25-30
207	Day nurse	1 week before	Had had moderate attack. Temp. 101-102. Had not yet regained strength	Throat injected. Tonsils ragged and inflamed. Slight glandular enlargement at angle of jaw	Had had tonsillitis once or twice within the past 5 years	Typical beta	50-60, almost pure culture
208	Patient	3 days before	Severe attack. Marked prostration. Temp. 102-103	Throat ulcerated. Moderate enlargement of glands of neck	Never had had tonsillitis before	Typical beta	Large numbers, almost pure culture
209	Patient	3 days before	Moderate prostration. Temp. 100-101	Throat injected and very sore. No ulceration. Moderate glandular enlargement	Subject to tonsillitis. Last attack 6 months before	Typical beta	Large numbers, almost pure culture

* 1 c.c. of a 24-hour broth culture injected intravenously into a rabbit caused high

but in no instance did a second attack occur in an individual who had had the disease in January.

The food of the patients and nurses was found to be satisfactory. The milk and cream were efficiently pasteurized. Furthermore, the incidence of the cases suggested contact infection, with a carrier as the causal agent.

Throat cultures were taken from all those who came in contact with the patients, including the cook, waitresses, nurses, attendants, etc. Blood-agar plates were made from the throat swabs by the same technic as heretofore described (Table 11). It was evident that the beta hemolytic streptococcus had caused the infection (see Chart 4). It was found in large numbers in the throats, of patients who were acutely ill, and in less numbers in the throat of a nurse who had had the infection recently, and in the throat of a nurse who had been a part of the epidemic 3 months previously.

Cases 198 to 203 (Chart 4) represent cultures from the employees who had had septic sore throat in January. At the time of study only one of these had the beta type of streptococcus in her throat—Case 201. Ever since the epidemic she had had trouble with the throat—tenderness and pain on swallowing, particularly in the morning. There was a slight enlargement of the nodes at the angle of the jaw. This nurse did not come into contact with all the patients, and but one of her patients had tonsillitis. She did come in contact with Nurses 204 and 207, and probably had infected them, one 3 weeks,

TABLE 11—*Continued*

CLINICAL CONDITION AND PRELIMINARY LABORATORY FINDINGS IN CONNECTION WITH POSITIVE CULTURES FROM THE B. HOSPITAL EPIDEMIC

Diameter of Colonies, mm.	Virulence for Animals (24-hr. broth culture)	Length of Chain (cocci)	Sugar Reactions							
			Dex-trose	Lac-tose	Mal-tose	Saccha-rose	Man-nite	Sal-i-cin	Raffi-nose	Inu-lin
2.5	0.5 c.c. killed mouse in 10 days (sticky peritonitis)	Long inter-lacing chains. 50-100 or more	2.3	2.0	2.0	2.1	1.1	2.0	1.2	1.2
2.5	0.5 c.c. killed mouse in 6 days	Long inter-lacing chains	2.5	2.4	2.6	2.3	1.2	2.3	1.1	1.0
2.5	0.5 c.c. did not kill mouse	Long inter-lacing chains, none shorter than 40-50	2.6	2.6	2.4	2.0	1.3	2.0	1.3	1.0
2.5	0.5 c.c. killed mouse in 7 days (sticky peritonitis)	Long inter-lacing chains. Few short chains. 8-12	2.4	2.0	2.3	2.2	1.0	2.5	1.2	1.3
2.5	0.5 c.c. did not kill mouse	Long inter-lacing chains. A few short chains	2.5	2.5	2.6	2.4	1.3	2.0	1.2	1.3

fever, followed in 5 days by polyarthritis.

and one 1 week previously. The patients in the ward did not begin to have tonsillitis until after Nurse 204 had recovered from tonsillitis and was back on duty. As night nurse she came into contact with all the patients, and probably was the source of the subsequent infection in patients 205, 206, 208, and 209. The latter two, who were acutely ill at time of visit, showed the presence of the beta hemolytic streptococcus in their throats in large numbers.

In summary: The beta hemolytic streptococcus may be found in the throat of an individual 3 months or more after an attack of septic sore throat. Such a carrier may be a source of infection for other persons, particularly if he comes into close personal contact with them.

CARRIERS. A STUDY OF THROAT CULTURES FROM 20 INDIVIDUALS WHO HAD HAD SEPTIC SORE THROAT WITHIN 2 YEARS PAST

The contact epidemic at the B. hospital strongly suggested that certain individuals harbor the beta hemolytic streptococcus in their throats for a long time after acute symptoms have subsided. This fact was also suggested by the continued presence of this streptococcus in

TABLE 12

CLINICAL AND PRELIMINARY LABORATORY DATA IN THE CASE OF PERSONS WHO HAD HAD SEPTIC SORE THROAT WITHIN 2 YEARS PAST

No.	Age	Time Elapsed Since Attack (mo.)	Degree of Infection	Present Condition	Hemolysis on Preliminary Plate			
					Macroscopic Appearance	Microscopic	Hemolytic Colonies per Plate	Diameter of Zone (mm.)
201B	25	3	Severe	Throat troublesome ever since attack	Beta type	Clear zone	10-15	2.5
99B	24	3	Severe Temp. 104. In bed 3 weeks	Throat normal apparently	Not beta type		40-50	1
17K	13	10	Moderate	Well since	Alpha type	Incomplete	20-30	
16K	12	10	Slight	Well since	Not beta type	Incomplete	10-12	
15K	18	10	Slight	Well since	Not beta type	Incomplete	4-5	
13K	14	10	Moderate	Well since			0	
12K	12	10	Slight	Well since			0	
11K	17	10	Moderate	Well since	Not beta type	Incomplete	8-9	3-4
7K	14	10	Moderate	Slow convalescence; well all last summer (1916)	Suggestive of beta type	Incomplete	6-7	5-6
3K	10	10	Severe	Well for past 6 months	Not beta type		3-4	
221	30	24	Severe	Well since	Not beta type		5-6	3-4
84N	3	8	Moderate	Well since			0	
82N	14	8	Severe	Well since	Suggestive of beta type	Complete	6-7	1.5-2
15D	7	8	Severe. Slow recovery	Not well since epidemic. 2-3 attacks of tonsillitis. Tonsils large, injected	Not beta type		75-100	0.5-1
21D	13	8	Severe. No complications	Well since	Not beta type	Incomplete	4-5	3-4
22D	10	8	Severe	Well since	Not beta type	Incomplete	8-10	1.5-2
23D	8	8	Severe	Tonsils large but not injected	Not beta type	Incomplete	4-6	1.5-2
24D	4	8	Severe	Well since	Not beta type	Incomplete	5-6	2-3
25D	6	8	Moderate	Tonsils ragged. No injection			0	
26D	14	8	Severe	Well since	Alpha type		15-20	

TABLE 13

RESULTS OF LABORATORY STUDIES OF STREPTOCOCCI SUGGESTIVE OF THE BETA HEMOLYTIC TYPE
FROM THE THROAT OF INDIVIDUALS WHO HAD HAD SEPTIC SORE THROAT
WITHIN 2 YEARS PAST

No.	Type of Hemolysis	Virulence for Animals (24-hr. broth culture)	Length of Chain	Sugar Reactions							
				Dex-trose	Lac-tose	Mal-tose	Saccha-rose	Man-nite	Sal-i-cin	Raffi-nose	Inu-lin
201B	Typical beta	0.5 c.c. killed mouse in 10 days	Long interlacing chains	2.5	2.0	2.0	2.1	1.1	2.0	1.2	1.2
83N	Not typical beta. Hemolysis complete but zone narrow	0.5 c.c. did not kill mouse	Long interlacing masses of chains	2.8	2.7	3.0	3.3	1.4	2.5	1.3	1.3
7K	Not typical beta. Zones 8 mm. Hemolysis incomplete	0.5 c.c. killed mouse in 3 days	Chains short, 8-10 some 20-30, but not longer	3.2	2.8	3.0	3.4	1.2	3.0	1.4	1.3

the throats of the boys of the K. boarding school after their temperatures had been normal for a week or two, and they had returned to their dormitories.

Twenty cultures were taken from the throats of persons who had had, within the past 2 years, unmistakable septic sore throat due to the use of contaminated milk. Any colonies on the original plate that were at all suggestive of the beta type of hemolysis were isolated in pure growth and their characteristics determined as regards reaction on carbohydrate media, length of chain, virulence for animals, etc.

The clinical data and preliminary laboratory findings are given in Table 12. Three cultures only were suggestive of the beta type of hemolysis (Table 13). Of these, only No. 201B was a definite beta hemolytic streptococcus. In this case, which has already been referred to in the discussion of the B. hospital epidemic, 3 months had elapsed at the time of study since the attack, and the patient during this time had suffered from irritation of the throat.

Chronic "irritation" of the throat after epidemic tonsillitis is not uncommon. In the K. boarding school, the attending physician of Case K3 writes: "Following the initial attack, the boy had chronic irritation of the throat, swelling of the glands of the neck, and was in a general run-down condition. Complete tonsillectomy three months after the infection resulted in ultimate recovery."

In summary: The greater proportion of cases of septic sore throat do not harbor the beta hemolytic streptococcus for a long time after recovery.

Certain individuals may retain the hemolytic streptococcus in the throat for 3 or 4 months after an attack of the disease.

TABLE 14
CLINICAL AND PRELIMINARY LABORATORY DATA IN CASES OF SPORADIC TONSILLITIS

No.	Age (yr.)	Day of Disease	Clinical Data		Complications	Type of Hemolysis
			Constitutional Symptoms	Local Condition		
365	12	2nd	Temp. 99.6. Moderate prostration	Throat injected. Slight exudate but no ulceration of tonsils	None.....	Not typical beta
366	9	2nd	Temp. 99.8. *Moderate prostration	Slight ulceration with exudate on tonsils	None.....	Suggestive of beta
367	30	3rd	Temp. 99-100. Slight prostration	Tonsils deeply injected. No ulceration	None.....	Alpha
368	5	4th	Temp. 99-100.....	Slight exudate with ulceration on tonsils	None.....	Beta
369	21	2nd	Temp. 100. Moderate prostration	Throat brilliantly injected. Moderate angina. Slight ulceration	None.....	Beta
370	9	6th	Temp. 101-102. Moderate prostration	Deep injection of throat. No ulceration	None.....	Beta
371	21	2nd	Temp. 99.6. Slight prostration	Tonsils injected but not ulcerated	Is subject to tonsillitis	Not beta
372	10	6th	Temp. 102-103. Severe prostration	Tonsils ulcerated with exudate. Glands of neck are tender	None.....	Beta
373	12	5th	Temp. 99-100. Moderate prostration	Throat injected. No exudate	Scarlet fever (?). Not possible to make a definite diagnosis	Beta
374	8	3rd	Temp. 101-102. Moderate prostration	Tonsils large and injected. Exudate on left	Septic sore throat 8 mo. before. Not well since	Not beta
375	1½	2nd	Temp. 101. Moderate prostration	Throat deeply injected. Exudate on tonsils. Moderate enlargement of glands of the neck	None.....	Not beta

THE PREVALENCE OF THE BETA HEMOLYTIC STREPTOCOCCUS
IN SPORADIC TONSILLITIS

Many observers have shown that acute follicular tonsillitis is associated with a variety of streptococci. Floyd and Wohlbach¹⁶ have demonstrated considerable variation in the cultural characteristics of such strains. In a few cases of sporadic tonsillitis we have applied to the flora from the throats the criteria set forth by Smith and Brown (Table 14).

Twenty cases were chosen, some severe, some moderately ill, and some only mildly so. Six blood-agar plates showed no hemolysis, or only pinpoint hemolytic colonies. Fourteen plates showed hemolytic colonies in large numbers, the diameter of the hemolytic zone varying from 2 to 6 mm. Many of the colonies were not even suggestive of

¹⁶ Jour. Med. Research, 1914, 29, p. 493.

TABLE 14—*Continued*
CLINICAL AND PRELIMINARY LABORATORY DATA IN CASES OF SPORADIC TONSILLITIS

No.	Age (yr.)	Day of Disease	Clinical Data		Complications	Type of Hemolysis
			Constitutional Symptoms	Local Condition		
376	18	3rd	Temp. 103-104. Severe prostration	Tonsils covered with exudate. Marked angina	Very slow convalescence	Beta
377	25	6th	Temp. 100-101. Moderate prostration	Tonsils large and injected. No exudate	Is subject to tonsillitis. Present attack a relapse from a more severe one	Not typical
378	18	6th	Temp. 100-101. Moderate prostration	Tonsils large and deeply injected. Exudate on right tonsil	None.....	Alpha
379	13	2nd	Temp. 102. Moderate prostration	Exudate with ulceration on tonsils. Moderate angina	Is subject to tonsillitis. Present attack a relapse	Beta
380	42	4th	Temp. 103-104. Severe prostration	Typical severe septic throat, with exudate, angina, etc.	None.....	Beta
381	4	8th	Temp. 99.6. Slight prostration	Slight ulceration of tonsils. Moderate enlargement of glands of neck	None.....	Suggestive of beta
382	6	5th	Temp. 101. Slight prostration	Throat deeply injected. Slight enlargement of glands of the neck	None.....	Not beta
383	10	3rd	Temp. 103-104. Prostrated	Tonsils ulcerated with heavy exudate	None.....	Beta
384	17	3rd	Temp. 101. Prostrated "Recovered in 3 days"	Tonsils ulcerated. Glands of neck tender and swollen	None.....	Not typical

the beta type, but they were isolated, and their cultural characteristics studied. A summary of these results is shown in Table 15.

Five of the 14 hemolytic cultures corresponded with the type described by Smith and Brown. The other 9 showed incomplete hemolysis, atypical carbohydrate reactions, or a low virulence for animals. Two of the 5 positive strains were from clinically moderate types of tonsillitis, Cases 370 and 379; 3 were from cases severe in type, Cases 372, 376, and 380. Case 380 was diagnosed septic sore throat by at least two clinicians, altho the case occurred sporadically. The more severe cases clinically were as a rule due to the beta type of hemolytic streptococcus; the more moderate cases usually were not. The streptococci from Cases 372, 380, and 381 were similar to the strain isolated by Smith and Brown from Outbreak A, the unusual characteristic being the absence of lactose-fermentation. The strepto-

TABLE 15
RESULTS OF STUDY OF CULTURES FROM 20 CASES OF SPORADIC TONSILLITIS

No.	Type of Hemolysis	Virulence for Animals (0.5 c.c. 24-hr. broth culture)	Length of Chain (cocci)	Sugar Reactions							
				Dex-trose	Lac-tose	Mal-tose	Saccha-rose	Man-nite	Salicin	Rafinose	Inulin
366	Not typical beta. Small Zone, 1.5 mm.	Killed mouse in 48 hours	8-12-20, seldom more	3.2	3.6	3.8	2.8	1.2	2.6	1.4	1.2
367	Suggestive of beta. Zone, 3-4 mm.	Did not kill mouse	40-50	3.7	3.4	3.2	3.3	2.3	1.3	2.4	1.3
368	Typical beta	Did not kill mouse	8-12, seldom longer	3.8	3.0	3.6	3.4	1.2	1.3	1.0	1.2
369	Beta type. Zones, 4-5 mm.	Killed mouse in 5 days	Very long and inter-lacing	4.0	3.4	3.3	3.4	2.8	1.4	4.1	3.1
370	Typical beta	Killed mouse in 3 days	30-50	3.0	3.1	3.7	3.0	1.0	2.7	1.3	1.0
371	Alpha	Did not kill mouse	Long and inter-lacing	2.8	2.5	2.0	2.2	2.4	0.9	1.1	1.2
372	Typical beta	Killed mouse in 48 hours	20-30, seldom longer	3.0	1.2	3.4	3.0	1.0	2.8	1.0	1.0
373	Typical beta	Did not kill mouse	8-12-20, seldom longer	3.4	3.0	3.6	3.2	1.0	1.2	1.1	1.0
376	Typical beta	Killed mouse in 36 hours	40-50	3.4	3.6	3.3	3.2	3.4	3.2	1.0	1.2
377	Not typical Hemolysis incomplete	Did not kill mouse	40-50 or more	3.0	3.1	2.7	3.4	1.3	3.0	1.3	3.4
379	Typical beta	Killed mouse in 36 hours	4-8-12, some 20-30, none longer	2.5	2.4	2.7	3.0	1.4	2.4	1.3	1.4
380	Typical beta	Killed mouse in 24 hours	4-8-12, seldom longer	2.6	1.3	2.5	2.4	1.2	2.4	1.2	1.1
381	Suggestive of beta	Did not kill mouse	4-8-12, not longer	2.5	1.0	2.4	2.5	1.2	2.4	1.3	1.2
383	Beta. Zones, 4 mm.	Did not kill mouse	2-4-8-12, not longer	4.3	3.7	4.2	3.5	1.2	3.6	1.1	1.2

coccus from Case 376 is similar to one strain described by Smith and Brown, in that it ferments both mannite and salicin.

In summary: Sporadic tonsillitis, severe or moderate, may be associated with the streptococcus of the beta hemolytic type described by Smith and Brown.

THE PREVALENCE OF THE BETA HEMOLYTIC STREPTOCOCCUS
IN SCARLET FEVER

There is a close relationship between septic sore throat and scarlet fever. Clinically, in septic sore throat, there is an acute onset, with high fever, brilliant injection of the nasopharynx, ulceration of, and false membrane on the tonsils, and in some instances a rash on the skin. In fact, the clinical picture is so similar to that of scarlet fever that in some epidemics part of the cases have been diagnosed as scarlet fever. The etiologic factor in epidemics of septic sore throat is carried by means of contaminated milk. The etiologic agent of scarlet fever may also be carried by milk.¹⁷ In April, 1915, in New York, and also in Dorchester, Massachusetts, there were epidemics of milk-borne septic sore throat, each traceable, not to a case of septic sore throat, but to a case of scarlet fever.

The following small epidemic brings up some interesting questions:

Three little girls had a tea party on Monday afternoon. The children were from different homes, were not in the same room at school, nor in any way exposed to similar conditions. There was no scarlet fever in the neighborhood. On Thursday afternoon all three developed sore throat. At the end of 2 more days, one child presented a typical picture of scarlet fever, with characteristic rash and all the other symptoms. One child had a mild sore throat, slight injection of the nasopharynx, and a mild and somewhat atypical rash. It was with considerable reluctance that the diagnosis of scarlet fever was made. The third child presented the typical picture of septic sore throat, with prostration, high temperature, ulceration of the throat, etc., but with no sign of a rash on the skin. From the throat cultures of each child the beta hemolytic type of streptococcus was isolated. The cultural characteristics, carbohydrate reactions, animal inoculations, and agglutination reactions of these three strains were exactly alike.

Were the first two children infected with something in addition to the beta hemolytic streptococcus, that produced scarlet fever in them? Is it possible that scarlet fever is due to 2 factors? Or did the third child really have scarlet fever without the rash?

It is certain that septic sore throat is due to a streptococcus. Is scarlet fever, after all, due to a streptococcus? Or, if scarlet fever is not due to a streptococcus, what part does the streptococcus play in the course of the disease? What type of streptococcus is found in scarlet fever? Does this type of streptococcus found in the throat of a scarlet-fever patient bear any relation to the mildness or severity of the disease?

METHODS OF STUDY AND RESULTS

Throat cultures from 48 cases of scarlet fever were examined for the presence of the beta hemolytic streptococcus. I have divided these cases into 5 clinical groups: Group I, 4 fatal cases of scarlet fever; Group II, 12 cases

¹⁷ Clark, Jour. Infect. Dis., 1915, 17, p. 109.

of "septic scarlet fever" (severe, with marked prostration, delirium, temperature of 104 F. or higher, ulceration of the throat, edema of the neck, etc.); Group III, 14 cases of moderately severe scarlet fever (with a maximal temperature of 102-103 F., exudate on the throat, but with slight or no ulceration, moderate degree of prostration, etc.); Group IV, 10 cases of mild scarlet fever (with typical rash but mildly injected throat without exudate, temperature 99-100 F., and no prostration); and Group V, carriers (who, tho recovered from the acute symptoms of scarlet fever, had developed a running nose, or discharging ears, the condition persisting for weeks or months).

A swab was taken of the throat and placed in 10 c.c. of normal salt solution. One 4-mm. loopful of this suspension was placed in a second 10-c.c. tube of salt solution, and one 4-mm. loopful of the latter suspension was placed in a 10-c.c. tube of horse-blood agar and plated at once. In this manner an approximate number of beta hemolytic colonies may be estimated, and compared with the numbers found on the plate at different stages of the disease, and in the different groups of cases.

The data are recorded in Tables 16 to 20.

Of 20 cases in which blood cultures were taken, including all the fatal and all the severe cases, only 2 yielded an organism from the blood—a beta hemolytic streptococcus—and in each instance the disease resulted fatally within 2 days.

The Fatal Cases.—In Table 16 are given the results of the studies of 4 fatal cases of scarlet fever. All the patients died in the 2nd week of the

TABLE 16
BETA HEMOLYTIC STREPTOCOCCI FROM FATAL CASES OF SCARLET FEVER

No.	Hemolytic Colonies	Virulence for Rabbits (24-hr. broth culture)	Length of Chain (cocci)	Carbohydrate Reactions								Time of Culture
				Dextrose	Lactose	Maltose	Saccharose	Man-nite	Sali-cin	Raffi-nose	Inu-lin	
19	Almost pure culture	0.5 c.c. caused high fever, septic joints, death	40-50	2.5	3.1	3.6	3.4	1.2	3.8	1.4	1.5	3 days before death
21	Pure culture	0.5 c.c. caused high fever, loss of weight. Recovery after 3 months	10-20, rarely 30	2.1	2.0	2.0	2.5	1.1	2.0	1.0	1.4	After death, from heart blood
35	Almost pure culture, 200-300	1 c.c. caused high fever, joint-involvement, paralysis and death (necrosis of vertebrae)	20-30, rarely longer	3.2	3.4	3.5	2.8	1.4	3.0	1.3	1.2	3 days before death
35	Pure culture	1 c.c. caused high fever, joint-involvement. Recovery in 3 mo.	20-30, sometimes more	3.0	3.2	3.1	3.0	1.2	3.4	1.4	1.4	After death, from heart blood
44	Almost pure culture	1 c.c. intravenously caused high fever, loss of weight, paralysis, and death	8-20-30, seldom more	3.4	2.3	3.1	3.4	0.9	2.1	1.0	1.1	2 days before death

TABLE 17

BETA HEMOLYTIC STREPTOCOCCI FROM SEVERE CASES OF SCARLET FEVER (TEMPERATURE 104 OR MORE, DELIRIUM, ULCERATION OF THE THROAT, EDEMA OF THE NECK, ETC., "SEPTIC SCARLET FEVER")

No.	Hemolytic Colonies	Virulence for Animals (24-hr. broth culture)	Length of Chain (cocci)	Carbohydrate Reactions								Day of Disease
				Dex-trose	Lac-tose	Mal-tose	Sac-charose	Man-nite	Salicin	Radi-nose	Inulin	
8	Large numbers, 200-300	0.5 c.c. intraperitoneally killed mouse in 48 hours	Long chains, 50-100	3.7	3.1	2.5	3.1	1.3	3.9	1.2	1.3	3rd
10	Large numbers, 200-300	0.5 c.c. intraperitoneally killed mouse in 24 hours	Long chains, 50-100	3.7	2.6	3.0	3.4	1.2	2.7	1.3	1.2	6th
12	5-6 colonies	1 c.c. in rabbit caused high fever, loss of weight, cardiac involvement. Recovered	60-100	2.3	2.0	2.3	2.9	1.1	2.8	1.1	1.0	24th
20	Almost pure culture, 200-250	1 c.c. in rabbit caused high fever, loss of weight, joint-involvement, death in 6 wk.	40-50	2.5	3.1	3.6	3.4	1.2	3.8	1.4	1.5	4th
24	Almost pure culture	1 c.c. in rabbit caused high fever, joint-involvement. Recovery	10-20, seldom longer	2.1	2.2	2.1	2.2	2.3	1.9	1.0	1.3	4th to 5th
23	Almost pure culture	1 c.c. in rabbit caused high fever, marked loss of weight. Recovery	30-40	2.5	2.6	2.6	2.3	2.1	3.7	1.0	1.3	4th
25	Almost pure culture, 200	1 c.c. in rabbit caused high fever, joint lesions. Recovery	30-40	2.5	2.3	2.6	2.7	3.2	2.9	1.0	1.3	5th
32	Almost pure culture, 200	1 c.c. in rabbit caused high fever, joint lesions. Recovery	Chains very and inter-lacing	3.8	4.0	4.4	4.3	1.3	3.0	1.6	1.3	10th
41	Almost pure culture, 200-300	1 c.c. in rabbit killed in 48 hours	30-40 or longer	2.3	3.7	3.8	3.4	3.7	3.3	1.5	1.5	4th
46	40-50	0.5 c.c. in mouse killed in 1 mo. (purulent pericarditis)	8-10-20, seldom longer	3.6	2.9	2.7	2.6	1.1	2.8	1.4	1.4	9th. Almost recovered when culture was taken
53	15-20	0.5 c.c. in mouse killed in 72 hours	8-10, sometimes 20-30	2.5	2.8	3.1	3.2	1.5	3.0	1.5	1.5	7th. Much improved on day culture was taken
70	Almost pure culture	1 c.c. intravenously in rabbit caused high fever, loss of weight. Recovery	Long chains, 50-80	2.0	2.2	2.1	2.2	1.2	2.4	1.2	1.2	8th. Still very septic

disease. In each instance, a typical beta hemolytic streptococcus was isolated from the throat, or from the blood, or both. Each streptococcus had high virulence for animals. These patients undoubtedly died from streptococemia.

The Cases of Septic Scarlet Fever.—Table 17 includes these cases. Beta hemolytic streptococci were present in these throats in almost pure growth.

TABLE 18

BETA HEMOLYTIC STREPTOCOCCI FROM SCARLET FEVER OF MODERATE SEVERITY (TEMP. 102-103, EXUDATE IN THROAT BUT NO ULCERATION, MODERATE PROSTRATION)

No.	Hemo-lytic Colo-nies	Virulence for Animals (24-hr. broth culture)	Length of Chain (cocci)	Carbohydrate Reactions								Day of Dis-ease
				Dex-trose	Lac-tose	Mal-tose	Sac-charose	Man-nite	Sal-i-cin	Raffi-nose	Inu-lin	
5	40-50	0.5 c.c. killed mouse in 36 hours	Long and inter-lacing	3.0	3.3	3.0	2.4	1.3	3.3	1.2	1.4	3rd
9	Almost pure cul-ture, 200	0.5 c.c. killed mouse in 4 days	40-50	4.0	3.8	4.0	4.1	3.8	4.1	1.3	1.4	3rd
13	200-300	0.5 c.c. killed mouse in 48 hours	Long and inter-lacing	2.6	3.4	3.0	3.1	1.5	1.4	1.2	1.7	4th
15	60-75	0.5 c.c. did not kill mouse	Long and inter-lacing	2.6	2.1	2.3	2.6	1.1	1.2	1.1	1.0	16th
17	4-5	0.5 c.c. did not kill mouse	30-40	2.4	2.1	2.3	2.3	2.2	2.2	1.1	1.1	2nd
22*	Almost pure culture, 150-200	0.5 c.c. killed mouse in 3 days	8-10, seldom more	2.1	2.2	3.0	2.4	1.0	2.3	1.2	1.3	7th
27	50-70	0.5 c.c. made mouse very ill. Recovered	2-4-8	2.8	2.9	2.4	3.1	1.6	1.1	1.3	1.1	10th
33	Almost pure culture, 150-200	1 c.c. in rabbit caused moderate fever, joint locali-zation. Recovery	30-40	4.2	4.0	4.5	4.5	3.1	4.8	1.5	1.5	4th
52	4-5	0.5 c.c. killed mouse in 3 days	20-30	2.5	2.6	2.9	2.9	1.6	2.4	1.6	1.6	3rd
54	40-50	0.5 c.c. killed mouse in 48 hours	40-50	2.3	2.7	2.5	2.6	1.5	2.6	1.5	1.4	3rd
55	2-3	0.5 c.c. killed mouse in 36 hours	20-30-40	2.9	2.7	2.8	2.7	1.5	2.7	1.8	1.7	3rd
57	8-10	0.5 c.c. did not kill mouse	Long and inter-lacing	5.5	5.2	5.2	6.4	1.2	4.8	4.1	4.7	4th
66	4-5	0.5 c.c. killed mouse in 10 days	Long and inter-lacing	2.0	1.8	2.3	2.2	2.1	1.3	1.6	1.3	8th
69	6-7	1 c.c. killed rabbit in 6 days	50-60	2.6	2.3	2.4	3.0	1.2	2.3	1.1	1.3	9th

* This case almost belongs in the severe-scarlet-fever group.

and in very large numbers from the 3rd to the 6th day of the disease, disappearing rapidly as the child recovered. With one exception the strains had high virulence for animals. A curve of the relative numbers of beta hemolytic streptococci in the throat of the child would correspond closely to its temperature curve or to any other index indicating the severity of the disease.

The Cases of Moderate Severity.—These cases are included in Table 18. All the throats contained streptococci of the beta type, but in much smaller numbers than in the cases of septic scarlet fever. As the symptoms of the child improved, there was a corresponding decrease in the number of streptococci in the throat.

The Cases of Mild Scarlet Fever.—Table 19 summarizes the characteristics of the strains of streptococci isolated from the throats of mild cases of scarlet fever. In the throats of only 2 of the 8 cases was the typical beta hemolytic streptococcus found, and here in comparatively small numbers. The virulence for animals of all the isolated strains was low, either not killing the mice at all, or causing death in from 7 to 10 days.

Carriers.—The carrier cases, Group V, are of great interest. These are the most dangerous to the community. Case 47 is typical. After a severe attack of scarlet fever, this patient developed a double otitis media. At the end of 9 weeks the discharge finally ceased, and the child was sent home. Two days later the ears began to discharge again, and 4 days later 2 members of the child's family developed severe scarlet fever. A streptococcus with identical characteristics was isolated from the throats of the patients with scarlet fever and from the ears of the child with otitis media. It has become

TABLE 19
BETA HEMOLYTIC STREPTOCOCCI FROM MILD CASES OF SCARLET FEVER (TEMP. 99-101, NO EXUDATE IN THROAT, NO PROSTRATION)

No.	Hemolytic Colonies	Virulence for Animals (24-hr. broth culture)	Length of Chain (cocci)	Carbohydrate Reactions								Day of Disease
				Dex-trose	Luc-tose	Mal-tose	Sac-charose	Man-nite	Salicin	Raffinose	Inulin	
16	Not typical beta	1 c.c. did not kill mouse	4-8-12	4.3	5.7	3.7	5.8	1.3	3.6	3.0	3.4	3 rd th
25	Not typical beta	1 c.c. did not kill mouse	30-40	2.7	1.3	2.4	2.0	2.4	3.0	1.2	1.0	4-5th
51*	30-50	0.5 c.c. killed mouse in 9 days	8-10 to 20-25	2.5	2.5	2.7	2.6	2.6	2.4	1.6	1.7	3rd
56	Not typical beta. 2-3 colonies	0.5 c.c. killed mouse in 4 days	30-40	3.5	2.5	2.4	2.7	1.4	2.4	1.3	1.4	3rd
58	Not typical beta. 2-4 colonies	0.5 c.c. killed mouse in 1 month (purulent peritonitis)	4-8, some 20	2.6	2.6	2.8	2.5	1.4	2.8	1.3	1.2	6th
63	20-40 colonies	0.5 c.c. did not kill mouse	4-8-12, some 20-30	3.7	3.4	3.6	3.2	2.0	3.2	1.2	1.4	6th
65	2-3	0.5 c.c. killed mouse in 7 days	4-8-12, some 30-40	2.2	2.0	2.3	2.3	1.3	2.4	1.1	1.2	7th
71	8-12	0.5 c.c. did not kill mouse	20-30	3.2	3.0	3.2	2.3	1.7	4.4	1.4	1.4	8th

* This case was more severe than the others included in this group.

so well established that children who have otitis media as a complication of scarlet fever are probably carriers of the disease, that no modern hospital for contagious diseases allows the return of these children to community life until all foci of infection have been healed.

TABLE 20
BETA HEMOLYTIC STREPTOCOCCI FROM CARRIER CASES OF SCARLET FEVER

No.	Hemo-lytic Colonies	Virulence for Animals (24-hr. broth culture)	Length of Chain (cocci)	Carbohydrate Reactions								Day of Dis-ease
				Dex-trose	Lac-tose	Mal-tose	Sac-charose	Man-nite	Salicin	Raffi-nose	Inulin	
8	12-14, nose	1 c.c. in rabbit caused high fever and joint-involvement. Recovery	40-50	2.4	2.5	2.1	2.2	1.1	3.1	0.9	1.0	48th
29	4-5, nose	0.5 c.c. intraperitoneally killed in 70 hours	Very long chains	2.6	2.1	2.9	2.2	1.1	2.7	1.2	1.0	56th
50	Pure culture, ears	0.5 c.c. killed mouse in 24 hours	20-30	3.2	2.7	2.4	3.0	1.4	2.6	1.3	1.4	34th
59	10-20, nose	0.5 c.c. killed mouse in 24 hours	8-12-16, seldom longer	2.4	2.7	2.7	2.3	1.0	2.5	1.4	1.4	129th
47	30-40, ears	0.5 c.c. killed mouse in 20 hours	25-30, no very long chain	2.2	3.2	2.4	2.3	1.4	2.3	1.4	1.6	70th
60	Ears none on original. Isolated from rabbit joint	0.5 c.c. killed mouse in 36 hours. 1 c.c. in rabbit gave high fever and joint-involvement	50-60	3.2	2.7	2.3	2.6	1.0	2.8	1.2	1.3	36th
61	Ears. None from original. Isolated from rabbit joint	1 c.c. in rabbit gave high fever, loss of weight, and joint-involvement	30-40	2.3	2.4	3.0	2.1	1.4	2.4	1.2	1.2	56th
62	300-350, ears	0.5 c.c. killed mouse in 36 hours (peritonitis and pericarditis)	40-50	2.4	2.0	2.3	2.2	1.0	2.1	1.4	1.5	16th
67	Ears. None from original. Isolated from joint of rabbit	1 c.c. in rabbit caused fever, loss of weight, and joint-involvement	30-40	2.3	2.4	2.7	3.2	1.6	2.5	1.4	1.3	90th
68	70-80 colonies, ears	1 c.c. in rabbit caused high fever, loss of weight, and death in 5 days	20-30, some inter-lacing masses	2.3	2.4	2.0	2.4	1.0	2.8	1.1	1.0	18th

Ten cases of the carrier type were studied. A summary of the results is given in Table 20. In each of the ten cases, a typical beta hemolytic streptococcus was isolated from the purulent material. If the infection was a recent one, as in Cases 62 and 68, the streptococcus was present in large numbers

and could be isolated with ease. In some of the long standing cases, 60, 61, and 67, it was found impossible to isolate a hemolytic streptococcus by means of the blood-agar plate because of a large number of secondary contaminating organisms that rapidly overspread the plate. Animal inoculation was at first unsuccessful; a large amount of the suspension of the purulent discharge caused death of the rabbits, but as a rule the rapidly spreading organism was also present in the blood culture; a small amount produced no symptoms. The interesting work of Faber¹⁸ offered a solution of the problem. He has shown that one can sensitize the joints of rabbits to various strains of streptococci.

Four rabbits were chosen that had had, 3 months previously, an intravenous injection of the beta hemolytic streptococcus. Each had developed the usual high temperature, with loss of weight and polyarthritis, but had finally recovered completely. Their blood still showed the presence of agglutinins for the beta hemolytic streptococcus. One 4-mm. loopful of the purulent material from each of Cases 60, 61, and 67, was added to 10 c.c. of salt solution and well shaken. The rabbits were injected with 0.5 c.c. each of this suspension. In from 4 to 6 days each rabbit developed an arthritis in the same joint that had been previously affected. Pure cultures of beta hemolytic streptococcus were isolated from the affected joints and studied.

Tho all the evidence seems to indicate that scarlet fever is not caused by the beta hemolytic streptococcus, nevertheless this strain plays a part in the course of the disease; in those cases that I have studied, the severity of the disease bears a close relation to the presence of, and also to the number of, the beta hemolytic streptococci in the throat. In rare instances a child develops "fulminating scarlet fever" and dies on the 2nd or 3rd day of the disease. This type of case probably succumbs to the actual etiologic agent of scarlet fever. Septic scarlet fever as seen in America, however, is, secondarily at least, a streptococcus infection, and in fatal scarlet fever, death is usually due to streptococcemia. These facts perhaps explain the results of Gabritschewsky¹⁹ and others with a streptococcus vaccine as a prophylactic against scarlet fever.

It seems not improbable that the beta hemolytic strain, which has been shown to be so persistent in cases of septic sore throat, may also be the factor which causes the chronic purulent discharges that so frequently follow scarlet fever. These results would seem to explain how it is possible for epidemics of septic sore throat to be caused by a case of scarlet fever. The discharges from a case of scarlet fever may contaminate milk either with the actual etiologic agent of scarlet fever, or with the secondary invader, or with both. Thus, contaminated milk may produce mild scarlet fever, septic sore throat, or septic scarlet

¹⁸ Jour. Exper. Med., 1915, 22, p. 615.

¹⁹ Centralbl. f. Bakteriöl., 1. O., 1906, 41, p. 719.

fever, depending on whether the individual is infected with one or both of these factors.

In summary: The beta hemolytic streptococcus was found in large numbers and in almost pure growth in the throats of all the severe cases of scarlet fever. It was not found in the throats of all the moderate or mild cases of scarlet fever, and when found occurred in less numbers.

The severity of a case of scarlet fever is usually directly proportional to the virulence of the beta hemolytic streptococcus found in the throat.

The chronic purulent discharges from the nose or ears of a case of scarlet fever may contain the beta hemolytic streptococcus for from 5 to 6 months after acute symptoms of the disease have subsided.

These results explain how epidemics of septic sore throat may be due to contamination of the milk supply with discharges from a case of scarlet fever.

FINAL SUMMARY

The epidemics of septic sore throat of Dorchester and the K. boarding school illustrate the fact that a hemolytic streptococcus may be found in the throats of the patients and also in the contaminated milk that caused the epidemic. This streptococcus, which seems to be a human strain, has definite characteristics by which it may be readily distinguished.

Persons with normal tonsils and normal mucous membranes of the nasopharynx do not harbor it in their throats except in rare instances. This fact probably explains the relative infrequency of contamination of milk, for the flora from normal human throats undoubtedly finds its way in many instances into the daily milk supply.

The beta hemolytic streptococci may be found in the throat for a long time after acute symptoms of septic sore throat have subsided. Individuals who have chronic "irritation" of the throat after an acute attack of septic sore throat are particularly apt to harbor the streptococcus and are therefore particularly dangerous to the community.

Sporadic tonsillitis may be due to the same streptococcus as is found in cases of septic sore throat. Clinically, it is often impossible to distinguish a severe case of sporadic, from a case of epidemic, tonsillitis; in all probability they are often the same disease. It is most likely that septic sore throat is kept alive in the community by means of the sporadic cases, or by means of carriers. Indeed, it seems most

probable that each infection with the beta hemolytic streptococcus is really an integral part of some epidemic. The infection may occur as an outspoken epidemic, as illustrated by the Dorchester outbreak, or it may be the result of contact infection, the mechanism of which is shown in the case of the B. hospital. It seems probable that cases which occur in interepidemic times, if their epidemiology could be traced, would usually prove to be a residual of some previous epidemic, which may, in turn, kindle a fresh epidemic. Every individual who harbors the beta hemolytic streptococcus for a long or short period of time is a danger to the community, in so far as he comes into intimate personal contact with his fellow men. Scarlet fever, in the severe and in the moderate type, is frequently accompanied by an infection with the beta hemolytic streptococcus, and this organism may remain in the discharges from the ears or nose of the patient for several months.

Epidemic tonsillitis, then, differs from sporadic tonsillitis due to this streptococcus in that the latter is an infection of the individual, usually by direct contact, and the former is an infection of the whole community, usually by indirect contact.

Septic sore throat, or epidemic tonsillitis, is not, therefore, a disease due wholly to the use of contaminated milk, and it is not necessary that the disease be present in the community in epidemic form, in order to make a definite diagnosis. The diagnosis can readily be made by comparatively simple laboratory procedures, and within a short period of time. The disease does not spread from one community to another in great epidemic waves, as do smallpox and influenza; its mode of spread resembles that of diphtheria or scarlet fever, and, as with these two diseases, the etiologic factor is probably more or less present in most communities of any size at all times, either in sporadic cases of the disease, or in the throats of carriers. Fortunately the individual who harbors this streptococcus is not of special danger to the community unless he comes into intimate personal contact with some of the activities of the community, as handling the milk supply.

CONCLUSIONS

A streptococcus was isolated from the throats of patients of the Dorchester epidemic of septic sore throat that was identical in its cultural characteristics with a streptococcus isolated from the suspected milk.

A streptococcus was isolated from the throats of patients in the milk epidemic at the K. boarding school that was identical in cultural

characteristics and agglutination reactions with a streptococcus isolated from the udder of one of the cows from the school dairy.

The types of streptococci isolated from the Dorchester milk epidemic, and the K. boarding school epidemic, are identical in their cultural characteristics and virulence for animals with the type of streptococci described by Smith and Brown in their studies of the milk epidemics of Massachusetts of 1914-1915. This type of streptococcus is a human strain, with a group of definite characteristics clearly described by Smith and Brown.

The streptococcus of this type is seldom found in normal throats (1% of the cases studied).

The throats of individuals who have had tonsillitis may harbor the beta hemolytic streptococcus for 3 or more months after acute symptoms of the disease have subsided.

This streptococcus is found in large numbers in the throats of a certain proportion of cases of sporadic tonsillitis (5 of 20 studied).

Epidemic tonsillitis, or septic sore throat, is, therefore, not a disease due wholly to the drinking of contaminated milk. The disease may exist not only in the epidemic, but in the sporadic form.

Tonsillitis, epidemic and sporadic, due to the streptococcus of the beta hemolytic type, may be severe or moderate, or even mild.

The beta streptococcus is frequently found in the throats of acute cases of scarlet fever, and may remain for months in the purulent discharges of the cases complicated by otitis media, etc.

These facts explain how the discharges from a case of scarlet fever may contaminate a milk supply and produce a milk-borne epidemic not of scarlet fever, but of septic sore throat.

TECHNIC

There is great diversity of opinion as to the proper media for study of streptococci. For the sake of uniformity and for comparative results we have followed closely the methods used by Smith and Brown.

Hemolysis.—Classification of types of hemolysis has been studied but little. The usual classification is either hemolysis, or no hemolysis, with occasional mention of incomplete hemolysis. The beta hemolytic streptococcus of Smith and Brown on horse-blood-agar plates forms a small lanceolate colony in the depths of the media. Around the colony is a clear-cut zone of hemolysis, from 2 to 4 mm. in diameter, at the end of from 24 to 48 hours. Under the low power of the microscope complete disappearance of the outline of

the red cells is noted. The border of the hemolytic zone is rather abruptly limited by the dense mass of red cells.

It is important to remember that the blood of different animals produces different types of hemolysis. Guinea-pig blood is particularly unsatisfactory. Dog blood produces a brownish discoloration. Washed corpuscles give a different type of hemolysis from that of whole blood, with narrower and less well-defined borders. Some of these peculiarities might be explained on the basis of acid-production by the organism. Cumming¹⁹ has shown a great variation in the hemolysis of the blood of different animals when weak solutions of acid and alkali are added to the blood. Many of the factors in the production of hemolysis, however, are not understood.

For the hemolytic plates, I have used 10 c.c. of veal infusion agar, +0.8 to +1, using Witte's peptone. The American peptones have produced unsatisfactory results in these studies.

The amount of blood is also important. One cubic centimeter of defibrinated horse blood is added to each agar tube. The plate is read at the end of 48 hours' incubation. The deep, and not the surface, colonies are taken as the standard. Results are not read if more than 50 colonies develop on the plate, since overcrowding of colonies produces noncharacteristic zones.

My experience has been that under similar conditions, a given organism, if freshly isolated (within from 2 to 3 weeks), will produce a definite and uniform type of hemolysis, and that the diameter of the hemolytic zone will be constant within a moderate latitude. For example, an organism will not have a 2-mm. zone at one time and a 4-mm. zone at the next subcultivation. These hemolytic characteristics change, however, in some instances after prolonged cultivation on artificial media.

Sugar Reactions.—The sugar reactions were studied by the generally accepted standard methods. Sugar-free veal infusion broth was prepared in 9-c.c. amounts, brought to +1, and sterilized. The carbohydrates were sterilized separately in the autoclave at 10 pounds' pressure for 30 minutes in 10% solution, and 1 c.c. of the 10% sugar solution added to each 9 c.c. of broth. All tubes were incubated for 24 hours to test sterility.

The carbohydrates used were dextrose, lactose, maltose, saccharose, mannite, salicin, raffinose, and inulin. Each tube was inoculated with 1 loop from a 24-hour broth culture of the organism to be studied and incubated for 7 days. The readings are all of titrations while hot, phenolphthalein being used as an indicator. Results are reported according to the usual method: that is, the number of cubic centimeters of normal NaOH which would be necessary to neutralize 100 c.c. of the broth.

This method of determining the reactions is subject to criticism. Since the work was begun, there have appeared several studies on the buffer reactions of meat infusion, and the determination of its true acidity by means of the electrolyte (Clark,²⁰ Bouvie,²¹ and others). I agree with the investigators that media should be standardized in relation to its hydrogen ionization rather than its titratable acidity. For the purposes of our study, however, the titration of the media has been just as satisfactory as the determination of the Ph.

In order to satisfy myself on this point, I chose 5 cultures of hemolytic streptococci from human throats and planted them in the sugar tubes. At the end of 7 days the tubes were titrated in the cold and then in the hot

¹⁹ Jour. Infect. Dis., 1916, 18, p. 151.

²⁰ Ibid., 1915, 17, p. 109.

²¹ Jour. Med. Research, 1915, 28, p. 295.

with phenolphthalein as the indicator. For titration in the work reported here freshly boiled distilled water was used. As the tables show, there is very little variation between the titrations in the hot and in the cold. The hydrogen-ion concentration was then determined in the same tubes, not by the electrolyte, but by the colorimetric method devised by Henderson, and used by Henderson and Palmer²² and their associates in the determination of the hydrogen-ion concentration of the urine (see Table 21).

The correspondence of the hydrogen-ion concentration with the titratable acidity is very close. The numbers of course represent the logarithms of the hydrogen-ion concentration and are a convenient method of expression. Thus 5.1 represents a greater degree of acidity than 7. The hydrogen-ion concentration of the control uninoculated tube was 7.2, or about the hydrogen-ion concentration of blood, which is 7.4.

TABLE 21
COMPARISON OF THE DETERMINATIONS OF ACIDITY IN MEDIA (ALL CULTURES ARE STREPTOCOCCI FROM HUMAN THROATS)

No.	Type of Hemolysis	Virulence for Animals (24-hr. broth culture)	Method of Titration	Sugar Reactions							
				Dex-trose	Lac-tose	Mal-tose	Sac-charose	Man-nite	Sal-icin	Raffi-nose	Inu-lin
56	Beta	0.5 c.c. killed mouse in 7 days	Titration cold.....	3.3	3.2	2.1	2.4	1.2	2.3	1.3	1.3
			Titration hot.....	3.5	3.5	2.4	2.7	1.3	2.4	1.5	1.6
			PH concentration...	5.1	5.5	5.4	5.5	7.2	5.7	7.0	7.2
57	Not typical beta. Incomplete	0.5 c.c. did not kill mouse	Titration cold.....	5.5	5.5	5.3	6.7	1.2	4.8	4.1	4.7
			Titration hot.....	5.6	5.7	5.4	6.8	1.3	5.0	4.2	4.9
			PH concentration...	4.5	4.4	4.6	4.4	7.4	4.7	4.8	4.7
58	Beta	0.5 c.c. killed mouse in 6 days	Titration cold.....	2.4	2.4	2.5	2.4	1.4	2.6	1.3	1.3
			Titration hot.....	2.6	2.6	2.7	2.5	1.5	2.8	1.4	1.5
			PH concentration..	5.4	5.5	5.6	5.6	7.2	5.5	7.2	7.0
59	Beta	0.5 c.c. killed mouse in 3 days	Titration cold.....	2.2	2.5	2.6	2.0	1.4	2.1	1.5	1.5
			Titration hot.....	2.3	2.7	2.7	2.3	1.6	2.5	1.7	1.7
			PH concentration..	5.7	5.5	5.6	5.8	7.2	5.7	7.2	7.2
50	Beta	0.5 c.c. killed mouse in 2 days	Titration cold.....	2.4	2.4	2.7	2.5	1.3	2.3	1.5	1.4
			Titration hot.....	2.5	2.6	2.9	2.7	1.5	2.4	1.8	1.6
			PH concentration..	5.4	5.5	5.3	5.4	7.0	5.6	7.0	7.0

Streptococci were planted in the sugar broths and incubated for 7 days. Each tube was titrated in the cold and then in the hot, phenolphthalein being used as indicator. The hydrogen ionization concentration of the same tube was then determined by the Henderson method.

Another point in the titration of media has caused no little discussion; namely, the amount of acidity which must be produced in a tube to indicate whether or not an organism splits a certain sugar. The streptococci which I have studied, if they have the ability to ferment a carbohydrate, do so in a definite and clear-cut way. The acidity in those tubes in which the sugar has been split is remarkably constant. On the other hand, the sugars which have not been acted upon give uniform end reactions. This fact has been more clearly brought out by determination of the hydrogen-ion concentration. Thus, for example, an organism that ferments both dextrose and salicin will not ferment dextrose to a hydrogen-ion concentration of 4.8, and salicin to 5.8, but both to about the same acidity. This suggests that a streptococcus will

²² Jour. Biol. Chem., 1912, 13, p. 363.

ferment a sugar until a hydrogen-ion concentration is reached which is incompatible with further activity of the organism. I have found as a general rule that the more pathogenic streptococci produce a less high acidity than the less pathogenic streptococci.

The Henderson colorimetric method for the determination of hydrogen-ion concentration is so simple, so accurate, and once the standard solutions are in stock, requires so little time, that I have found this method of determining the acidity of media a very practical one.

Virulence for Animals.—The virulence of the streptococci isolated from septic sore throat has been studied in many of the reported epidemics. The most complete study is that of the Chicago epidemic by Jackson.¹⁵ The author produced severe polyarthritis in all the rabbits reported. The exact technic is not given. Smith and Brown injected rabbits with 1 c.c. of a 24-hour broth culture intravenously. We have followed this method. Temperature and weight charts were kept of each animal. This procedure enabled us to pick out the mildly pathogenic organisms, for in some instances the only reaction was an increased temperature for 3 or 4 days. It also made it possible to prognosticate joint-involvement, for a rise in temperature usually preceded the tenderness and swelling in each fresh joint.

When mice were used, 0.5 c.c. of a 24-hour broth culture was injected intraperitoneally. An organism is not called pathogenic for mice unless the mouse died, and the streptococcus was recovered from the peritoneum or heart blood. The pathogenicity for mice of 0.5 c.c. of a 24-hour broth culture intraperitoneally is a fair index of its pathogenicity in rabbits. Comparative results proved that in all the cases studied, a streptococcus which killed a mouse in from 3 to 4 days would produce high fever and marked loss of weight in rabbits, followed usually by polyarthritis, when 1 c.c. of a 24-hour broth was injected intravenously. For the preliminary studies mice were often used. In substantiation of the more important cultures rabbits were used for confirmatory evidence.

Capsule-Formation.—Previous reports, particularly those of Davis and Rosenow,⁸ emphasize the importance of the capsule as a distinguishing characteristic of the streptococcus isolated from milk epidemics. This characteristic is not emphasized by Smith and Brown. The capsule of this type of streptococcus is not a true capsule but a capsular substance. All the beta hemolytic streptococci studied by us when freshly isolated have had a capsular substance, in some instances very little, in others more, in all cases disappearing rapidly on cultivation. Capsular substance, therefore, and also length of chain, are so variable and of so little differential importance that they are not reported in these studies.

Agglutination.—Twenty-four-hour broth cultures were shaken in a mechanical shaker for 15 minutes, then allowed to stand for 1 hour. The living suspension was added to the serum dilutions in small test tubes, the total fluid in each tube being 2 c.c. The tubes were placed in the incubator at 37.5 C. for 1 hour, then placed in the refrigerator and read at the end of 24 hours. C indicates complete clumping and sedimentation; + + + +, complete clumping without complete sedimentation; + + +, clumping nearly complete; + +, partial clumping; +, slight; ±, doubtful, and —, no clumping.

THE BACTERIOLOGY OF PEMPHIGUS NEONATORUM *

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There have occurred in and about Chicago within the last year 5 small epidemics of the disease commonly known as pemphigus neonatorum.

The first epidemic that was brought to my attention occurred at West Side Hospital in November, 1915. Only 3 cases developed, none of which was fatal.

The second epidemic, involving 5 babies and 1 nurse, occurred at the University hospital. There were no deaths.

The third epidemic occurred at the Cook county hospital in July, 45 cases appearing in the maternity ward. The first case occurred in the child of a woman who had been admitted with an impetigo of the skin around the mouth. Other cases rapidly developed, 2 of which terminated fatally. From cultures from several of these cases a staphylococcus was isolated which yielded a light-yellow growth on plain agar and was markedly hemolytic on plain blood agar. Smears from the vesicles revealed a kidney-shaped diplococcus, intracellular and extracellular, and a great preponderance of polymorphonuclear leukocytes in the fluids.

Several of the mothers developed lesions on the breasts and upper part of the body and one nurse developed a lesion. The ward had to be closed for over a month before the disease could be stamped out.

After this epidemic had developed, several women waiting for confinement at the county hospital were sent to the Monroe Street hospital. None of them was infected, either at the time of entrance into the Monroe Street hospital or subsequently. However, they transmitted the disease to about 9 children with whom they came in contact, all of whom were over 2 years of age. Nursing babies and bottle-fed babies in the same institutions, but not in contact with these women or infected children, remained free from the disease.

The fourth epidemic appeared at the Chicago Lying-In hospital, four babies being affected. There were no deaths. Staphylococcus aureus was found.

The fifth epidemic occurred at the Englewood hospital in September. There were 12 cases. Diplococci, intracellular and extracellular, were seen in smears from the vesicles. One baby died, but its death was said to have been due to causes other than pemphigus.

A peculiar epidemic of a disease the clinical description of which closely resembles that of pemphigus neonatorum occurred this summer in a camp for boys at Culver, Indiana. About 10% of 900 boys between the ages of 10 and 20 years were affected with a vesicular skin eruption, which was accompanied by no constitutional symptoms, and which, under white precipitate ointment, cleared up rapidly without scar-formation.

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Opinions differ concerning the pathologic significance of organisms found in the lesions of this disease. The staphylococcus and streptococcus have been declared by most writers to be the causative organism.

Demme¹ (1886) was the first to cultivate an organism from a case of acute contagious pemphigus. This was a nonchromogenic diplococcus.

Almquist² in 1891 isolated an organism from a series of cases of pemphigus neonatorum, which was a diplococcus when studied in the serum of the vesicles of the disease, but which closely resembled *Staphylococcus aureus* when grown on artificial media. When inoculated into the human skin, however, it showed no tendency to produce the deep infection or carbuncle characteristic of *Staphylococcus aureus* infection, but produced the vesicular eruption typical of pemphigus neonatorum. He concluded therefore, that in spite of its close resemblance to the staphylococcus in cultural characteristics, it was a different organism, and he suggested the name *Micrococcus pemphigi neonatorum*.

Matzenauer,³ after histologic and bacteriologic examination of both impetigo contagiosa and pemphigus neonatorum, concluded that they were identical. He believed that the organisms which he had isolated were indistinguishable from *Staphylococcus aureus*.

In 1900 Sabouraud⁴ divided the cases of this disease into 2 main divisions or classes—the vesicular type of Tillbury Fox, and the pustular type of Bochart. He based his conclusions more especially on his bacteriologic observations with a special technic. He classified the cases of pemphigus neonatorum as of the vesicular variety and asserted that they are due to a streptococcus. Later the lesions, in his opinion, become secondarily infected with a staphylococcus, which has been wrongly supposed by most investigators to be the cause of the disease. In practically all cases in which the streptococcus was isolated, ascitic fluid had been used as the culture medium, and for cultures the contents of the vesicles had been obtained in the early stages of their development. He lays great stress on the cultural value of the liquid media. He obtained a mixture of staphylococci and streptococci when he used ascitic fluid and broth in equal parts. With plain broth he found the staphylococcus in almost pure culture. On solid media he invariably obtained the staphylococcus. These results he explains on the ground that the initial lesions of the disease are due to infection with the streptococcus, emphasizing the rapidity of incidence of the lesions as a point in favor of his view. Some hours or days subsequent to the initial infection the lesion becomes infected with the staphylococcus which in the later stages is found in pure growth in the lesions.

There are several points about the work of Sabouraud which need further elaboration before his views can obtain recognition. In the first place, he did not reproduce the lesions by the injection into other patients of cultures of the streptococcus. Secondly, as he himself points out, the media that he used for growing the streptococcus has an inhibiting action on the staphylococcus. In cases in which a culture medium was used which was favorable to both organisms, he always obtained a more luxuriant growth of the staphylococcus. This organism, moreover, was always present together with the streptococcus in smears from the lesions. It is difficult to see how Sabouraud can advance as the etiologic agent of a disease an organism that

¹ Verhandel. d. Cong. f. inn. Med., Wiesbaden, 1886.

² Ztschr. f. Hyg. u. Infektionskrankh., 1891, 10, p. 253.

³ Virchow-Hirsch Jahrb. d. ges. Med., 1900, 25, p. 549.

⁴ Sabouraud, Ann. de dermat. et de syph., 1900, 31, p. 325.

has fulfilled but one of Koch's laws. Granting that a streptococcus may be present early in these cases, the fact that the staphylococcus is also present renders quite unwarranted any conclusions from this fact alone, as to which of the two is the primary and which the secondary invader. Furthermore, the fact that the staphylococcus found in connection with this disease fulfills all of Koch's laws, indicates that this organism is the cause of the disease. Finally, Sabouraud gives no description of the cultural characteristics of the streptococci. Inasmuch as staphylococci may under certain circumstances appear in short-chain formation, this point should be elucidated.

Block⁵ in 1900 described 15 fatal cases, giving good pathologic reports. He found a streptococcus in the heart blood in several cases, but believed it to be a secondary invader. In the skin lesions he found *Staphylococcus albus* and *Staphylococcus aureus*, and a coffee-bean-shaped diplococcus.

Clegg and Wherry⁶ in 1906 isolated from cases of pemphigus neonatorum occurring in the Civil hospital at Manila, a diplococcus corresponding to those described by Almquist, and closely resembling *Staphylococcus aureus* on culture media, but showing some features which they considered distinctive. They found in addition to Almquist's findings that litmus milk was coagulated in about a week. No indol was produced or cholera red in Dunham's broth containing 0.01% KNO_3 , after 10 days. In a 1% glucose broth solution containing $\frac{1}{2}$ part sterile goat serum, growth appeared with remarkable rapidity, a tube being densely clouded, while control tubes, inoculated with *Staphylococcus aureus* and *Sarcina lutea*, showed only a faint growth. With the formation of acid the serum was precipitated as a dense flocculent mass. No gas was found in 1% glucose, lactose, and saccharose broth; cloudiness appeared in both open and closed arms of the fermentation tubes.

Morphologically the organisms in preparations of agar and broth were indistinguishable from pyogenic staphylococci. In media prepared from milk, or better, serum broth, the diplococic arrangement found in smears from the vesicle contents was well reproduced. Chromogenic characteristics were better brought out on gelatin and glucose agar than on plain agar. One cubic centimeter of a 48-hour broth culture injected into a guinea-pig intraperitoneally, caused no reaction in 1 week. Small amounts of the same serum broth culture injected under the skin of a rabbit gave rise to no vesicles and caused only small hyperemic areas, which disappeared in a week. Self-inoculation on the forearm of one of the investigators gave a typical lesion in 30 hours, but the organism was not recovered. There was no subjective sensation, except a slight itching, and resolution occurred in 48 hours without scar-formation.

Max Neisser⁷ considers Almquist's organism a strain of the staphylococcus. The organism corresponds exactly with the description given by Neisser of typical *Staphylococcus aureus*. However, he reports no work with Almquist's organism in support of his contention.

The epidemic of pemphigus neonatorum which I have investigated occurred at the University hospital, Chicago, in February, 1916.

There had been no previous cases in the practice of any of the members of the obstetrical staff. There had been an epidemic at West

⁵ Brit. Jour. Dermat., 1900, 12, p. 304.

⁶ Jour. Infect. Dis., 1906, 3, p. 165.

⁷ Kolle and Wassermann's Handb. d. pathogen. Mikroorganismen, 1912, 4, p. 389.

Side Hospital, distant one block, a few months before the outbreak in question, but since the attending and nursing staffs of the two institutions are entirely distinct, no avenue of communication from that source could be traced. As far as could be ascertained no cases of *inpetigo contagiosa* had been treated in the hospital immediately preceding the outbreak of the epidemic. Midwives are not allowed in the hospital, but as the greater number of obstetrical cases in the University hospital are Russian Jews living in the Ghetto, the possibility of an examination of patients by midwives previous to their entry to the hospital cannot be denied, altho no history to that effect could be elicited.

None of the mothers of the babies was suffering from leukorrhea, and none ran a temperature during the puerperium. There were 6 cases altogether and a description of one typical case will suffice for all.

The eruption appeared on the 8th day on the flexor surface of the left arm at the bend of the elbow. The initial lesion was a macula, which enlarged by peripheral extension and became pale in the center. Soon the skin began to rise in the center of the lesion, forming a minute vesicle with a peripheral ring of hyperemia. The epidermal covering of the lesion was very thin and transparent. The fluid contents were clear, but later became turbid as the lesion developed into a bulla. The lesion spread with remarkable rapidity in an excentric manner, so that in 24 hours it was as large as a dollar, and answered in every respect the description of the smaller vesicle which preceded it. As the lesion enlarged the fluid contents became turbid, and the thin epidermal covering was wrinkled and flaccid. Other vesicles of a similar character rapidly appeared on various parts of the body, especially on the arms and thighs. Rupture of the vesicles and bullae occurred at various stages of their development, disclosing a hyperemic base, moist and glistening. In some instances the vesicles spread peripherally after the rupture. They usually, however, tended to remain stationary or to heal rapidly subsequent to rupture. There were no general manifestations of the disease. The children nursed well and ran no temperature. The leukocyte count was slightly raised, averaging 15,000 white corpuscles, with red corpuscles normal. A differential count was unfortunately not made.

Cultures were made both aerobically and anaerobically on blood-agar media and growth was obtained in both cases, but the organism grew much more luxuriantly under aerobic conditions. Anaerobic and aerobic transplants to plain agar resulted in the same luxuriant aerobic and faint anaerobic growth as was seen in the original cultures.

The attempts that have been made to inoculate this organism into animals with a view to reproducing the disease have been uniformly unsuccessful. Rabbits and guinea-pigs have been used, and subcutaneous, intradermal, and intraperitoneal injections have been reported

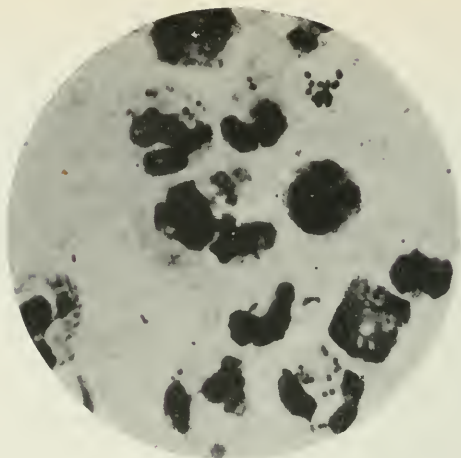


Fig. 1. Smear from vesicle before rupture, showing preponderance of polymorphonuclear leukocytes and intracellular and extracellular diplococci. One short chain is also seen. Methylene blue stain. $\times 1200$.

by Clegg and Wherry, who report negative results except for hyperemia at the site of some of the subcutaneous injections. However, they used relatively small doses (1 c.c. of 48-hour broth cultures), and they do not mention how long the organism had been cultivated on artificial media before it was used in the animal experiment. Since this organism apparently had been the cause of death in children affected with the disease, I determined to make further tests of its pathogenicity in lower animals. In the first experiment a rather large dose was employed intraperitoneally to determine roughly its virulence.

A 24-hour blood-agar slant culture in 5 c.c. of sterile normal salt solution was injected intraperitoneally into a half-grown guinea-pig. The animal remained perfectly well for 3 days; then, an edematous tender swelling occurred over the lower abdomen. The animal refused to eat, and death occurred the next day. There was marked edema of the subcutaneous tissues and skin with hemorrhages into the skin and underlying muscles of the abdominal walls. This condition was most marked at the site of the injection, and spread around to the back and down both hind legs.

Adhesions and subperitoneal hemorrhages were noted on opening the peritoneal cavity. There was an abscess the size of a split pea in the spleen, with perisplenic adhesions, and an ulcer 2 mm. in diameter at the pylorus of the stomach. The liver and kidneys were normal; the gallbladder was distended. There was a hemorrhagic infarct of the right lung. Heart and pericardium were normal.

Cultures from the skin, muscles, and heart blood were made on blood agar and the causative organism recovered in pure growth in all cases.

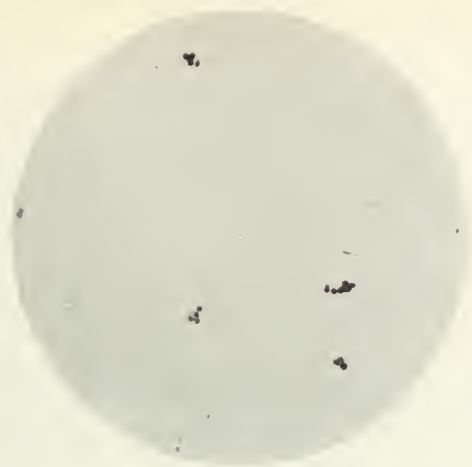


Fig. 2. Smear from a 48-hour broth culture. One short chain is shown and the tendency toward staphylococcic clumping. Some of the diplococci are kidney-shaped. Gram stain, $\times 1200$.

It was thought that by intravenous injection the elective affinity of the organism for the skin might be demonstrated.

A half-grown rabbit was injected intravenously with 2 c.c. of a heavy suspension of the organism in salt solution. The animal appeared sick the next day and did not eat. It developed diarrhea, and died on the 3rd day. Autopsy revealed an abscess of the kidneys, an ulcer, 1 cm. in diameter, at the pylorus of the stomach, pneumonia in both lungs, acute cholecystitis, hematorachis of bright blood underneath the pia. There were no metastatic skin lesions.

Because of the nature of the organism and because of its tendency in most human cases to remain localized in the skin, tho capable of causing severe manifestations and death on gaining access to the blood stream, it was determined to inject some animals subcutaneously and some intraperitoneally to determine whether there were differences in behavior under the given conditions. As the infection runs a more severe course in children than in adults, young guinea-pigs were used as lending themselves more favorably to the conditions of the experiments.

In all cases 1 c.c. of a heavy suspension of organisms grown on blood agar for 24 hours was used intraperitoneally for injection. These organisms had been isolated from the lesions in patients 6 days previously and had been grown on blood agar in the interval.

SERIES 1

Guinea-pig 1.—Died the next day. Autopsy: Peritonitis, cholecystitis, perisplenitis, and salpingometritis. Heart, lungs, spleen, liver, and kidneys

normal. Cultures from the peritoneal cavity and the heart blood gave, from the former, a luxuriant growth of staphylococcus in pure culture in 24 hours, and from the latter, a few colonies of the same organism.

Guinea-pig 2.—Appeared well for 4 days following the injection; then there developed paresis of both hind legs, more marked on the right side. Animal could move them somewhat when painful stimuli were applied. Paresis increased. Death 15 days after inoculation. No clinical evidence of peritonitis. Autopsy: Broncho-pneumonia in both lungs with adhesive pleuritis, adhesions in the peritoneal cavity, and evidence of recent peritonitis, no skin abscesses found. Spinal cord showed rather extensive subpial hemorrhages near the cauda.

Guinea-pig 3.—No symptoms until 5 days later when a tender edematous swelling appeared on the under surface of the abdomen and the animal seemed



Fig. 3. Guinea-pig showing paralysis of hind legs, which developed 4 days after an intraperitoneal injection of 1 c.c. of a heavy suspension of the organism grown on blood agar for 24 hours.

ill. Death 15 days later without any change in the clinical picture. Autopsy: Generalized peritonitis, lobular pneumonia, and acute nephritis. Liver, spleen, gallbladder, and heart normal. No skin lesions.

Cultures from the heart blood and the peritoneum revealed the organism injected.

SERIES 2

Guinea-pig 1.—Appeared sick and died in 7 days. Autopsy: Marked edema and cellulitis at the site of injection, and local peritonitis just under the skin lesion. Adhesive peritonitis appeared about the stomach, pancreas and spleen. Small amount of clear serous fluid in the peritoneal cavity. No skin lesions except at the site of injection. Cultures from heart blood and peri-

cardium on blood agar yielded the organism injected in luxuriant growth. Skin culture negative.

Guinea-pig 2.—Gradually sickened and was found dead in 7 days. Autopsy: Abscess on right side and enlarged subcutaneous lymphatic glands near the inguinal ring on the same side. The abscess was 1 cm. across, with ulcerations of the skin and formation of bright-yellow pus. Peritonitis below the abscess on the right side, with adhesions to the ileum; no generalized peritonitis. Pneumonia of right lower lobe. Heart, liver, spleen, and kidneys normal.

From these experiments it will be seen that the organism in question is virulent, producing severe lesions and death in guinea-pigs and rabbits. Even in the cases in which subcutaneous injections were made, the tendency to invasion of the deeper tissues and to systemic infection is clearly seen in positive peritoneal and blood cultures. In Series 2 particular pains were taken to avoid injecting any bacteria into the peritoneal cavity. There was no tendency of the organisms to localize in the skin in any of the experiments. This speaks strongly for the view that the disease is an infection by contact with contaminated material and not a systemic infection in the early stages of the disease, as has been suggested by many authors.

The peculiar tendency toward hemorrhages into the spinal cord and paralysis or paresis in some of the animals is worthy of note. Further experiments are in progress to determine whether this is a frequent feature, or merely accidental in these cases.

Because of repeated failures of many observers to produce lesions in rabbits and guinea-pigs by intracutaneous injections an attempt was made to reproduce the lesion in a monkey.

A young *Macacus rhesus* monkey not being obtainable, a young Java monkey was inoculated intradermally. A drop of a 24-hour broth culture was placed on the tender skin of the inner surface of the upper arm after cleansing with 95% alcohol. Then, with a rather coarse needle, the skin was pierced through the drop in such way that the needle ran almost parallel to the skin surface and just raised the epidermis, without penetrating the dermis. An abortive vesicle resulted in 48 hours, which after 72 hours was excised and sectioned. The sections showed an elevation of the epidermal layer and some leukocytic infiltration of the underlying base. The whole lesion was abortive in type, measuring not more than 2 mm. in diameter. There was no erythematous areola as seen in lesions in human cases. A control inoculation with the sterile needle made in the same way was negative.

Reports of auto-inoculation experiments have been made by Almquist and by Clegg and Wherry. The typical lesions were reproduced in each case, but in neither of these experiments was an attempt made to recover the organism from the lesion. It was in an endeavor to do

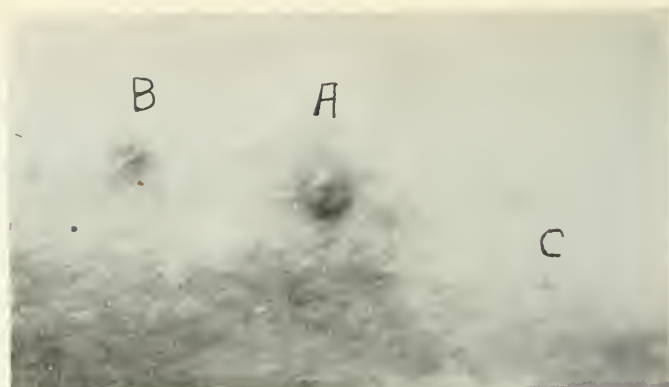


Fig. 4. *A* indicates a typical lesion on flexor surface of the forearm produced 36 hours after intracutaneous auto-inoculation. Note the darkened area surrounding the vesicle, denoting the hyperemic aureola, and also the characteristic wrinkling of the covering of the lesion. *B* indicates the hyperemic area just preceding the development of the vesicle at that point. *C* indicates the site of the control inoculation with sterile needle.

this and thus fulfill Koch's postulates with regard to this organism that the writer decided to inoculate himself.

The flexor surface of the left forearm was carefully scrubbed with 95% alcohol and most of the superficial epidermis was removed with the object of getting rid of staphylococci present on the skin surface.

A 24-hour culture in Dunham's peptone solution was used which had been inoculated from a Loeffler's serum culture after the organism had been on artificial medium for 3 weeks. One drop of the peptone solution was placed on the cleaned flexor surface of the forearm and a slight prick was made through the skin and down to the corium. The needle was withdrawn through the drop, and without sterilizing was inserted into the skin in a similar manner about 4 cm. away from the site of the primary injection. A control inoculation was made with sterilized needle about 4 cm. from the second inoculation.

After 4 hours, and again after 8 hours, nothing was noted at the site of inoculation. In 15 hours an erythematous area, 0.5 cm. in diameter, appeared at the site of the second inoculation, and in 20 hours a distinct vesicle appeared. It was the size of a pea, yellowish in color, with a very thin, wrinkled covering of epidermis, which was transparent. The vesicle was surrounded by an area of hyperemia about 3 mm. wide. The site of the primary inoculation had become red and hyperemic at this time, but no vesicle had appeared. The control inoculation was negative.

After 36 hours the first vesicle had increased in size to 7 mm. and the yellowish fluid contents had become turbid. The covering was more wrinkled and less tense than formerly. At this time the second vesicle was beginning to form on the hyperemic area at the site of the primary inoculation.

In 48 hours the first began to decrease in size, altho it had not yet ruptured. The second had increased to 5 mm. in diameter, and rested on an areola 2.5 cm. in diameter. It answered in all respects the description of the primary vesicle. The control remained negative. There were no subjective sensa-

tions. A blood count made at 48 hours showed 9000 white cells. A differential count gave 58% polymorphonuclear neutrophils, 13% large mononuclear, and 27% small mononuclear leukocytes, 1% eosinophiles, and 1% basophiles, 200 cells being counted.

At 48 hours the tops of both vesicles were removed and cultures made from each on Loeffler's medium by means of a platinum loop. Some of the fluid contents of the vesicles were taken up by means of a capillary tube and smears made from this. The culture from the first vesicle, which, as mentioned, was beginning to undergo retrogression, was sterile. Cultures from the second, which was at the height of development, yielded a good growth of the organism injected.

Smears from the vesicles showed intracellular and extracellular diplococci, which were gram-positive, and morphologically resembled the kidney-shaped diplococcus described by Almquist. A differential count of the white cells in the vesicle contents revealed a high percentage of lymphocytes. This is the opposite of what Almquist found in the lesions of babies; namely, predominance at this time, of polymorphonuclear leukocytes.

Following the removal of the thin transparent covering of the lesion, the base was very hyperemic, reddened, moist, and glistening. It was tender to the touch and smarted when alcohol was applied. The edges were even and round. On the 4th day after inoculation the areola had disappeared, and the bases of the lesions were a deeper red. There was a slight tendency to the reformation of the vesicle at the edges of the lesion and to further peripheral extension. There were no subjective sensations.

A large scale came from the first lesion on the 7th day, and from the second vesicle on the 10th day after inoculation, leaving a shiny smooth base, which in a week was indistinguishable from the surrounding integument.

Reports in the literature of control experiments with *Staphylococcus aureus* are numerous. Thus Garré⁸ rubbed a pure culture on the uninjured skin of his arm and in 4 days developed a large carbuncle with a surrounding zone of furuncles. An even more analogous experiment was that of Bockhart⁹ who suspended a small portion of an agar-agar culture in sterile salt solution, and scratched the suspension gently into the deeper layers of the skin with the finger nail. A furuncle developed at the site of inoculation.

Culturally, the organism from pemphigus neonatorum cannot be distinguished from many strains of staphylococcus. Its reactions on the various media are given in the accompanying tables, together with its fermentation reactions and its ability to produce acid in sugar solutions.

As to the thermal death point, this organism closely resembled the other strains of *Staphylococcus aureus*. Agar tubes were inoculated and kept at 60, 65, 70, 75, and 80 C. for 10, 20, and 30 minutes. The organism was able to withstand 60 C. for one-half hour, but 65 C. for 10 minutes killed all but an occasional organism.

⁸ Fortschr. d. Med., 1885, 3, p. 165.

⁹ Monatsh. f. prakt. Dermat., 1887, 4, p. 450.

On plain-blood-agar plates the organism was strongly hemolytic. The colonies appeared gray and semitranslucent, and did not become pigmented.

This strain produced indol, as do other strains of the staphylo-

TABLE 1
CULTURAL CHARACTERISTICS OF THE ORGANISM FROM PEMIPHIGUS NEONATORUM

Media	24 Hours	48 Hours	72 Hours	1 Week
Plain agar...	Moderate growth; slightly spreading and raised at edges, glistening, smooth, translucent; no odor, discoloration, or pigment	Slight yellow pigment	More pigment	Pigment fairly well marked; faint musty odor
Plain broth..	Diffuse turbidity, some deposit	Increased turbidity, more gray deposit	Same.....	Dense turbidity, moderate yellowish deposit
Litmus milk	Less alkaline.....	Less alkaline....	Less alkaline..	Acid; no coagulation; blue precipitate at bottom
Gelatin.....	Faint cup-shaped depression	Marked depression	More liquefaction	Liquefaction almost complete
Potato.....	Scant, slightly raised, confined to streaks, faintly butyrous; faint musty odor	More growth, more pigment, media slightly darkened	More growth, media discolored	Strong yellow pigment; media darkened
Loeffler's blood serum	Beaded at edge, deep-yellow, slightly raised, glistening, butyrous; faint musty odor	Same.....	Same.....	Same
Russell media	Top layer reddish, intermediate layer yellowish, deep layer blue and red	Same changes but more marked	Completely acid	No change
Levulose....	Good stab growth, slight surface growth	Increased surface growth	Same.....	Light-yellow, moderate surface growth
Lactose.....	Good stab, poor surface growth	Increased surface growth	Same.....	Light-yellow surface, good stab growth
Inulin.....	Good stab, fair surface growth	Increased surface growth	Same.....	Luxuriant surface growth, orange-yellow, gray at edges
Salicin.....	Slight surface, good stab growth	Increased surface growth	Same.....	Luxuriant surface, moderate stab growth, orange-yellow
Raffinose....	Slight surface, good stab growth	Increased surface growth	Same.....	Luxuriant surface growth, orange-yellow; moderate stab growth

coccus. It differs in this respect from the organism described by Clegg and Wherry.

From these data it would appear that the causative organism of this disease is one that culturally and biologically is identical with the staphylococcus. Morphologically, on certain media it differs slightly

in that it appears as a diplococcus and occasionally forms chains. Pathologically, it differs in that it produces a lesion that is peculiar to this type of infection.

These differences do not seem sufficient for considering the pemphigus coccus as a different species. It would seem more correct to regard it as a strain of the staphylococcus with certain peculiarities as to cultural and pathogenic properties which differentiate it from other strains of the same organism.

The name pemphigus neonatorum is an unfortunate one for this disease. True pemphigus has been looked on as a severe constitutional disease, attacking principally older people, and as a disease in which a serious prognosis is usually made. The etiology is obscure in the

TABLE 2
SUGAR-FERMENTATION AFTER 3 DAYS

Sugar	Growth in Fermentation Tubes		Gas		Percentage of Acid Formed
	Open Arm	Closed Arm	24 hr.	48 hr.	
Lactose.....	++	++	0	0	.8
Saccharose.....	++	++	0	0	.875
Maltose.....	+++	+++	0	0	.85
Dextrose.....	++	0	0	0	.80
Mannite.....	+	+	0	0	.75
Raffinose.....	+	+	0	0	.50
Inulin.....	+	0	0	0	.45
Salicin.....	+	0	0	0	.40
Control.....	0	0	0	0	.40

Phenolphthalein used as indicator.
N/10 NaOH used for titration.

extreme, no causative organism having thus far been described. In pemphigus neonatorum we are concerned with an entirely different type of disease. There are few or no constitutional disturbances, the infection being limited to the skin, and the causative organism has been isolated in pure culture, and fulfills Koch's laws. It would therefore seem advisable to classify this disease pathologically under a different heading; namely, dermatitis. I suggest that the name epidemic staphylococcic vesicular dermatitis of the newborn, be applied to the disease.

SUMMARY

Pemphigus neonatorum is a peculiar type of staphylococcic dermatitis occurring in the newborn, but capable of transmission to adults.

The causative organism is a strain of *Staphylococcus aureus*, indistinguishable culturally and biologically from some other strains of

staphylococcus, but differing under certain circumstances morphologically, and showing different pathogenic tendencies.

This organism has fulfilled all of Koch's laws with respect to the disease. Typical lesions from which the organism has been recovered have been produced in man. It is pathogenic for lower animals, but injections have thus far failed to reproduce the specific disease.

The epidemic nature and possibly fatal termination of the disease make its early recognition and active treatment highly desirable.

In view of the clinical and experimental data it appears that the infection spreads by contact with infected material and that the portal of entry is the intact skin.

In keeping with its etiology and pathology, the name epidemic staphylococcic vesicular dermatitis of the newborn is suggested for this disease.

STUDIES ON IMMUNITY IN TYPHUS EXANTHEMATICUS WITH REFERENCE TO THE ANTIBODIES IN MAN AND GUINEA-PIG DEMONSTRABLE BY THE DALE METHOD*

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In the studies by Plotz, Olitsky, and Baehr¹ on the etiology of typhus fever, one of us reported the occurrence of agglutinins, complement-fixing bodies, precipitins, and opsonins in the blood of typhus-fever patients. It is significant that all types of antibodies occurred regularly after the crisis, occasionally at the crisis, and only rarely before the crisis. These antibodies usually persisted in the blood many months after convalescence. In the blood of the guinea-pig, to which the disease is transmitted by the injection of human virus, no antibodies except opsonins could be demonstrated.

The present paper is concerned with the study of the serum of patients and of guinea-pigs suffering or recovering from typhus fever, by a method involving the anaphylactic response. This method was first used by Dale² in the study of anaphylactic phenomena. It depends on the muscular contraction of the uterus of a sensitized guinea-pig when brought in contact with the antigen. Briefly, the technic of all such experiments is as follows: The uterus is removed from the animal and suspended in a container filled with Locke's solution, kept at a temperature of 37-40 C.; it is attached to a lever which writes on a moving drum. The antigen is added to the Locke's fluid bathing the uterus, and a contraction is recorded by the up-stroke of the lever. Ergamine is used to test the contractility of the muscle. This method was first successfully applied to the study of infectious diseases (pneumonia) by Weil.³

Antigens prepared by various methods were tried. The following proved the most satisfactory. The 7-day anaerobic growth on 0.5% glucose serum agar of *Bacillus typhi-exanthematici* was emulsified in physiologic salt solution,

* Received for publication October 12, 1916. Work done under tenure of George Blumenthal Jr., fellowships in pathology.

¹ Jour. Infect. Dis., 1915, 17, p. 1.

² Jour. Pharmacol. and Exper. Therap., 1913, 4, p. 167.

³ Weil and Torrey, Jour. Exper. Med., 1916, 23, p. 1.

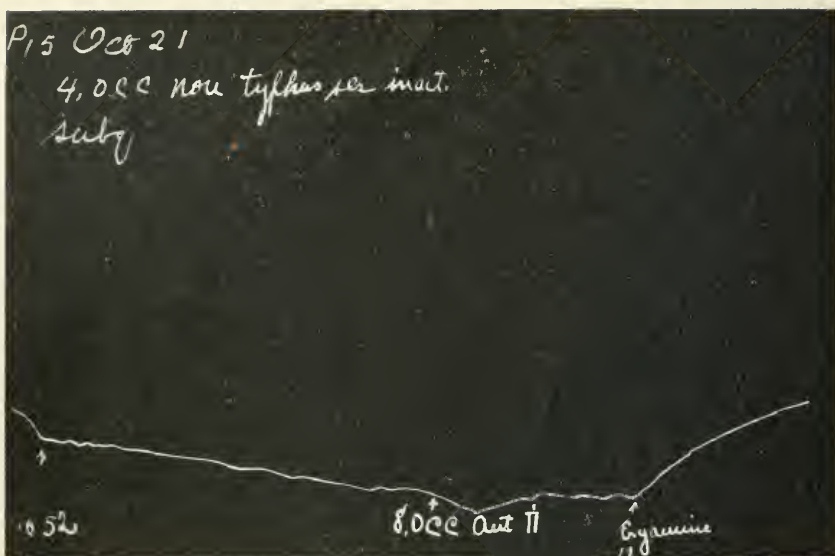


Fig. 1. The titration of the antigen. Guinea-pig 15 received 4 c.c. of inactivated non-typhus serum subcutaneously on October 18. On October 21, the uterus was tested; no response to 8 c.c. of antigen. Approximate dose of antigen 4 c.c.



Fig. 2. The titration of the antigen. Guinea-pig 44 normal. No reaction to 5 c.c. of antigen.

washed 6 times in 0.9% salt solution, and finally suspended in distilled water. This suspension was incubated for 24 hours at 37 C., sealed in sterile tubes, and kept on ice. Many substances, human, horse, and rabbit sera, when applied in sufficient dosage to the guinea-pig uterus suspended in a Dale apparatus, cause a contraction by virtue of their irritant action. Each antigen was therefore tested against the uterus of a normal guinea-pig to determine the largest dose that would fail to induce a contraction. One-half this amount or less was used in the experiments on injected animals (Figs. 1 and 2).

The antigens were also tested against the uteri of guinea-pigs injected with normal serum. It will be recalled that *Bacillus typhi-exanthematici* is grown on human-serum (ascitic fluid) media; even the most thorough washing may fail to eliminate traces of serum clinging to the bacterial bodies used as antigen. Reactions between traces of serum in the typhus antigen and the antibodies developed in the guinea-pig against the injected human serum must therefore be excluded. Antigens which gave a positive response were discarded and only those used which failed to cause a contraction in the uteri of guinea-pigs injected with normal serum. In the end the complications arising from the possible presence of human serum in the antigen were altogether eliminated by the production of typhus fever in guinea-pigs and by the testing of such animals with antigens prepared as described.

The antigen was also tested for its antigenic properties—its capacity for reacting to specific antibodies. This was demonstrated in the following way: Rabbits were immunized against the typhus bacillus by the repeated injection of living organisms and 10 days after the last injection, the animals were bled. Two cubic centimeters of this immune serum were injected subcutaneously into female guinea-pigs and after an interval of 3 days, the uteri were tested for response to the antigen. The contraction was in each case striking, indicating the capacity of the antigen to react to antibodies developed for *Bacillus typhi-exanthematici*.

ANTIBODIES IN PATIENTS WITH TYPHUS FEVER

The experiments with the serum of typhus-fever patients will be discussed first.

Blood was taken from the patient by venepuncture, and after the separation of the clot, the serum was removed. From 3 to 5 c.c. were injected subcutaneously into virgin female guinea-pigs. The sera from the first 7 patients were injected fresh (without being inactivated). It was found, in agreement with the experience of Schloss, that fresh serum is toxic for guinea-pigs, causing either death or local ulceration or infiltration. The uteri of these animals failed to react on the addition of antigen to the surrounding Locke's fluid; this was due probably to the failure of absorption of the toxic fresh sera. This series of 7 cases was therefore discarded.

The sera of the 4 remaining cases were inactivated; with these sera, 7 experiments were made.

Two typical experiments will be described in detail. On October 29, Guinea-pig 23 received 4.5 c.c. of inactivated serum obtained from Case B59, 9 days after the crisis. On November 2, the guinea-pig was killed and the uterus suspended in the Dale apparatus. Figure 3 shows the reaction to 3 c.c. of antigen, indicating that the guinea-pig had absorbed the typhus antibodies from the injected serum. It is noteworthy that a second addition of 3 c.c. of antigen

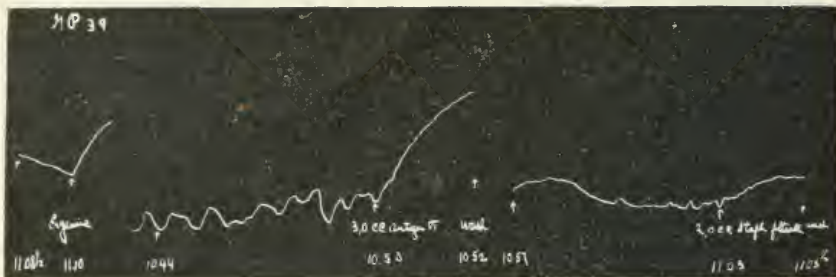


Fig. 3. Reaction to postcritical typhus serum. On October 29, Guinea-pig 23 received 4.5 c.c. of serum of Case B59, 9 days after crisis. November 2, uterus tested. Note reaction to 3 c.c. of antigen; also failure to react on the second addition of antigen, indicating desensitization. Good reaction to ergamine.

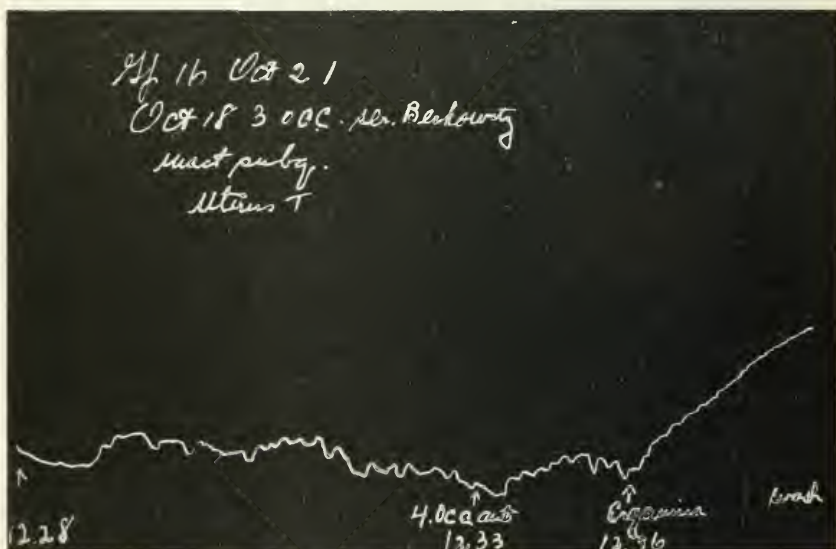


Fig. 4. Absence of reaction with typhus serum at height of disease. On October 18, Guinea-pig 16 received 3 c.c. of inactivated serum of Case B59. On October 21, uterus tested. Note absence of reaction to 4 c.c. of antigen.

failed to produce a contraction; the uterus had been desensitized by the first dose. The response to ergamine shows that the uterus retained its contractility.

Figure 4 illustrates the result of injecting serum from the same case at the height of the disease. On October 18, Guinea-pig 16 received 3 c.c. of inactivated serum obtained from Case B59 on the 10th day of illness at the height of the disease. There is no reaction to 4 c.c. of antigen, altho there is a sharp response on the addition of ergamine.

Of the 7 experiments in this series, 3 were made with sera obtained during the height or febrile stage of the disease (1st to 10th day). All were negative, indicating the absence of demonstrable antibody in the serum of typhus-fever patients during the height of the disease. In the remaining 4 experiments sera obtained from 4 to 9 days after the crisis were used. Three of these reactions were positive, indicating the presence of antibody in the postcritical period of typhus fever. The negative reaction occurred with serum sent to us from another hospital and in this case the possibility of a mistaken diagnosis exists. The significance of these findings will be discussed later.

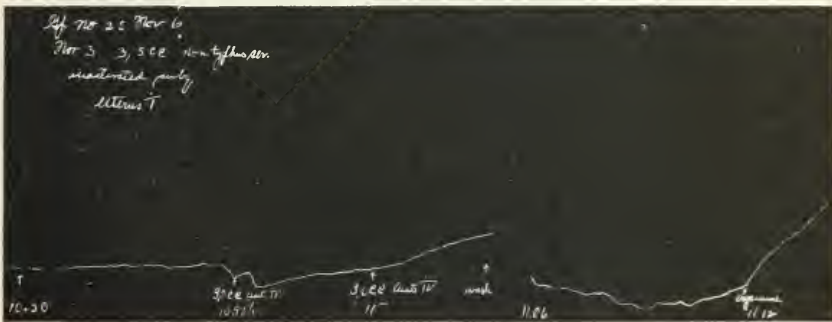


Fig. 5. Absence of reaction with nontyphus serum. On November 3, 3.5 c.c. of nontyphus serum injected subcutaneously. Note absence of reaction to 3 c.c. and almost negligible reaction to an additional 5 c.c.

As a control to these experiments, the blood of 6 individuals not suffering from typhus fever was tested (Figs. 2 and 5). Two of these were normal people, the remaining four were patients suffering from various febrile conditions, influenza, liver abscess, etc. The technic was identical with that used in the experiments with typhus-fever serum. There was a complete absence of uterine response in every case. Figure 5 illustrates a typical case. On November 3, Guinea-pig 25 received 3.5 c.c. of inactivated nontyphus serum subcutaneously. Three days later the animal was killed and the uterus was suspended in the Dale apparatus. There was no reaction to 3 c.c. or to 5 c.c. added shortly afterward. The reaction to ergamine indicated that the uterus retained its contractility.

CELLULAR ANTIBODIES IN GUINEA-PIGS

Ricketts and Wilder, in 1910, showed that the virus of typhus fever can be transmitted to guinea-pigs; that after an incubation period of from 7 to 10 days, the temperature rises, remains elevated for a period of from 7 to 14 days, and that recovery usually follows. Re-inoculation with typhus virus fails to cause a febrile reaction, thus indicating the development of immunity. In his studies on such immune guinea-pigs, Olitsky reached the following conclusion: "Altho the animal was proved immune, yet the serum contained no agglutinin or complement-fixing bodies. . . . It is most probable that the guinea-pig reacts to the typhus virus and develops subsequently a high grade of immunity by means of its tissue elements and only to a very slight degree by means of the circulating blood." If the immunity is indeed chiefly of a cellular nature, the Dale method is peculiarly fitted to the study of the immune process in the guinea-pig. The uterine-muscle preparation of a guinea-pig sick of typhus fever demonstrates by its contraction on the addition of typhus antigen, the existence of antibodies in the cells against *Bacillus typhi-exanthematici*.

The *modus operandi* of these experiments was as follows: Female virgin guinea-pigs were injected intraperitoneally with the virus of typhus fever. This virus was obtained originally from a case of endemic (New York) typhus fever in the wards of Mount Sinai Hospital in 1911. It has been kept alive in guinea-pigs since that time. The virus (which must have passed thru approximately 150 generations in guinea-pigs) has maintained its strength with remarkable uniformity; it regularly produced a temperature of about 6 days' duration after an incubation period of about 10 days when inoculated intraperitoneally into guinea-pigs.

Fourteen guinea-pigs, not including the controls, comprise this series. The animals were tested during the height or febrile stage of the disease and at varying intervals after the crisis. Two typical experiments will be outlined in detail.

On November 18, Guinea-pig 39 was injected intraperitoneally with typhus virus from Guinea-pig 353. After an interval of 10 days, it developed a temperature which remained elevated for 5 days. Five days after the crisis, the animal was killed and the uterus suspended in the Dale apparatus. Figure 6 shows a marked reaction on the addition of 3 c.c. of antigen. The contraction following ergamine indicates that the uterine muscle retained its contractility.

Figure 7 illustrates an experiment on a guinea-pig killed during the height of the disease. Guinea-pig 51 was injected intraperitoneally January 1 with 3 c.c. of typhus virus. January 13, the 3d day of fever, the animal was killed and the uterus was suspended in a Dale apparatus. On the addition of antigen there was a complete absence of response, and the contraction following ergamine indicates that the uterus retained its contractility.

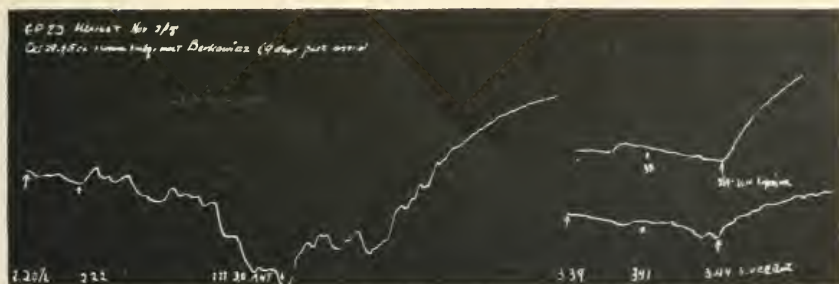


Fig. 6. Reaction in typhus-virus guinea-pigs after the crisis. Guinea-pig 39 showed typical febrile reaction to injection of typhus virus. Five days after the crisis, uterus tested. Note marked reaction to typhus antigen, negligible reaction to staphylococcus antigen, and good reaction to ergamine.



Fig. 7. Absence of reaction in typhus-virus guinea-pigs during the height of the disease. Guinea-pig 51 showed typical febrile reaction to typhus-fever virus. At the height of the fever, uterus tested. Note absence of response to antigen and good reaction to ergamine.

Of the 14 guinea-pigs in this series, 5 were tested during the height of the disease. Of these five none showed a reaction on the addition of antigen. Of the remaining 9, tested at varying intervals after the crisis, 7 showed a positive reaction. One of 2 guinea-pigs killed post-critically that failed to react, was killed only 2 days after the crisis. The seven guinea-pigs that showed a muscular response on the addition of antigen, were tested 3, 5, 8, 10, 12, and 18 days after the crisis.

To determine whether the reaction obtained in guinea-pigs convalescent from typhus fever was due to a specific response to the addition of typhus antigen, several antigens made from other organisms



Fig. 8. Reaction to typhus antigen in typhus-virus animals after crisis, and failure to respond to typhoid antigen. Guinea-pig 66 reacted in typical manner to typhus virus. Uterus tested 8 days after the crisis. Note reaction to typhus antigen; also absence of response to typhoid antigen. Reaction to ergamine marked.

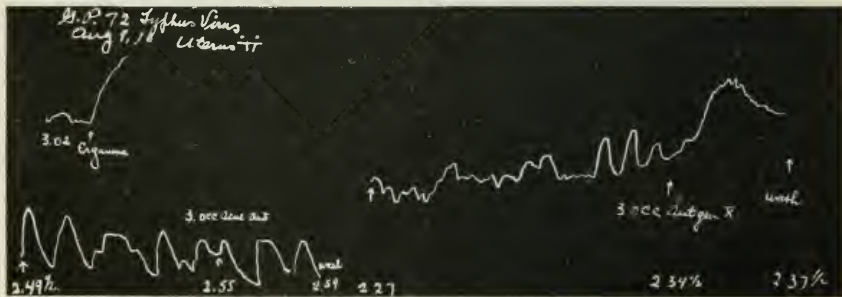


Fig. 9. Reaction to typhus antigen in typhus-virus animals after crisis and failure to respond to acne antigen. Guinea-pig 72 reacted in typical manner to injection of virus. Eight days after crisis uterus tested. Note reaction to 3 c.c. of typhus antigen and failure to respond to acne antigen. Reaction to ergamine good.

were used. Thus, antigens were made from *M. aureus*, *B. typhosus*, and *B. acne* by the method used in preparing typhus antigen. For the strains of *B. acne* we wish to express our indebtedness to Dr. E. P. Bernstein. The antigens were first tested against normal uteri to ascertain the correct dosage. Figures 6, 8, and 9 show the absence of response to the antigens mentioned, and repetition of the experiments confirmed these findings. The negative results with *B. acne* are particularly significant because of the slight similarity of the morphology of this organism to that of *B. typhi-exanthematici*. These results tend to corroborate the previous findings in regard to the specificity of the typhus bacillus in typhus fever.

DISCUSSION

The experiments thus detailed establish the fact that an anaphylactic antibody can be demonstrated in the serum of typhus-fever patients after the crisis and is not demonstrable during the height of the disease. This agrees with the finding of Olitsky that agglutinins, opsonins, and complement-fixing bodies are rarely present during the febrile stage of the disease, but are uniformly to be found after the crisis. From these facts one can not assume the entire absence of antibodies in the circulation during the febrile period. Weil has suggested, as a result of his work on unformed proteid substances and also on the blood of pneumonia patients, that other factors must be taken into consideration; that the presence of antigen (and in infectious diseases that means the specific microorganism or its derivatives) may prevent the demonstration of the antibody, which may nevertheless be present. These theories are particularly suggestive in relation to typhus fever, as Plotz has found the organism in the blood of patients 2 days after the crisis, when antibodies were already present in the serum.

It will be recalled that the previous workers have inferred that the immunity of guinea-pigs to typhus fever acquired in the initial attack must be ascribed to the development of cellular antibodies, because antibodies in the circulating blood could not be found. The present work demonstrates the presence in guinea-pigs of cellular antibodies against *B. typhi-exanthematici*. Here, also, the antibody could not be shown to exist during the febrile stage of the disease, but occurred only after the crisis.

CONCLUSIONS

Antibodies against *Bacillus typhi-exanthematici*, demonstrated by the Dale method, are found in the serum of typhus-fever patients after the crisis. These antibodies are not present in the serum during the height of the disease.

Antibodies against *Bacillus typhi-exanthematici* are found in the cells of typhus-fever guinea-pigs after the crisis. These antibodies are not demonstrable during the height of the disease.

The reaction in the serum of typhus-fever patients and in the cells of the guinea-pig is specific; that is, by the use of similar methods no antibodies to *Bacillus typhi-exanthematici* could be demonstrated in the

blood of normal individuals or of patients suffering from other infections. Guinea-pigs in the postcritical stage of typhus fever showed antibodies only to *Bacillus typhi-exanthematici*, and not to *Micrococcus aureus*, *Bacillus typhosus*, or *Bacillus acne*.

The results with the Dale method offer further evidence of the etiologic relationship between *Bacillus typhi-exanthematici* and typhus exanthematicus.

A SMALL CONTACT EPIDEMIC OF TYPHOID FEVER *

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The growing realization of the importance of the transference of infection in typhoid fever through contact, both direct and indirect, especially in the maintenance of the disease in rural communities, prompts the report of the following outbreak.

Maurice is a small Iowa village of about 250 inhabitants. It is chiefly a trading point and place of residence for retired farmers. It does not have either a municipal water supply or a municipal system for the collection and disposal of sewage and garbage. Water is obtained from numerous shallow wells. Excreta are disposed in privy vaults.

Until July, 1916, Maurice had been free from typhoid since 1914; at that time a small epidemic had occurred, apparently due to contact. Subsequent to July 1, 1916, 9 cases of typhoid fever occurred in persons living in Maurice, and 2 in residents of the adjoining country side, a morbidity rate of 360 per 10,000 of population.

During the course of the investigation 2 cases of typhoid fever in the neighboring town of Orange City were investigated and their relationship to the present outbreak established. Some time after the completion of the investigation, it was found that there were 3 additional cases related to the present outbreak, one in Maurice, one in the country side, and one in a child living in Monroe, South Dakota.

By weeks the onset of cases was as follows: First week, beginning July 2, 1 case; 2nd week, 2 cases; 3rd week, 7 cases; 4th week, 2 cases, and 5th week, 1 case.

The distribution of the cases according to age and sex was as follows:

Sex	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	Total
Males	1	4	1	0	1	0	1	1	9
Females	2	3	0	0	0	0	0	0	5
	<hr/> 3	<hr/> 7	<hr/> 1	<hr/> 0	<hr/> 1	<hr/> 0	<hr/> 1	<hr/> 1	<hr/> 14

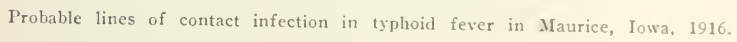
Patients 1, 3, 4, 5, 11, and 12, residing in Maurice, had not been outside the town. Patient 2, brother of Patient 1, had been in Orange

* Received for publication October 12, 1916.

City 2 weeks before he became ill. He had also been in Hawarden and Sioux Center on the same date. Patient 7, a school girl, living in Maurice, had gone to the country for a brief visit and had there become ill after a few days. Patients 6 and 9, living in the country adjacent to Maurice, were in Maurice 12 and 16 days, respectively, before the onset of their illness. Patients 8 and 13, school children living in Orange City, were in Maurice 16 and 26 days before the onset of their illness. The out-of-town patients were in Maurice in the period between the 3rd and the 5th of July. It therefore seems clear that the residents of Maurice contracted the disease within the town and that the out-of-town cases had been in Maurice at a time when they might have received infection. Furthermore, from a study of the out-of-town cases, it would appear that infection must have been contracted early in July.

Patient 1 represents the first case of the outbreak. Patients 2 and 3, her brother and father, developed the disease in the next week. Both had been in contact with her during the early part of her illness. Patient 4, the grandfather of Patient 1, had been with her in the period preceding July 5th, under circumstances that would give plenty of opportunities for the transference of infection. Patients 5, 7, 11, and 12 were playmates of Patients 1 and 2, and had been in contact with them about the time of the onset of illness in Patient 1. Patient 10 was an intimate friend of Patient 4 and had visited with him during the early stages of his illness. Patient 6 had been in contact with Patient 3 at the store of the latter early in July. Patient 9 is a niece of Patient 3 and had been at the home of Patients 1, 2, and 3 on the day before Patient 1 became ill. Patients 8 and 13, living in Orange City, had been at the home of Patients 1, 2, and 3 between the 3rd and the 5th of July, the latter date marking the onset of illness in Patient 1. Patient 14, living in Monroe, South Dakota, had also been at this home on July 3. It is evident, that, the first patient excluded, all those developing typhoid fever subsequently had been in contact with the cases in Patients 1, 2, and 3, or with cases arising therefrom. This is shown in the accompanying diagram.

A consideration of the possibilities for infection through foods, flies, ice cream, milk, drinking water, and swimming, shows that none of these factors was common to all the patients. Patients 1, 2, 3, 4, 5, 8, 9, and 13 had consumed well water from the premises of Patients 1, 2, and 3. Examination of this water showed it to be highly con-



Probable lines of contact infection in typhoid fever in Maurice, Iowa, 1916.

taminated, tho it is not clear from what source excremental material could gain entrance. But the manner in which the cases developed, and the fact that the water was not a factor common to all the cases, together with all other circumstances of the epidemic, indicate that infection was passed directly from person to person, rather than through the indirect agency of infected water.

It is not clear from what source Patient 1 contracted the disease, since she had not been outside of Maurice before her illness. She had been in close contact with 4 persons who had had typhoid fever within recent years, 3 of them during 1914 in the small epidemic previously mentioned, which was apparently due to contact. These individuals were her mother, older sister, cousin, and a distant cousin, who had had typhoid fever 4 years before while in Arabia. During June, 1916, the mother had been confined to her bed by an ill-defined sickness, the chief characteristics of which had been pleurisy and thrombophlebitis. A few years previously she had had a panhysterectomy performed because of carcinoma, and during June had suffered from a recurrence, from which she died during September, 1916. During her illness in June, she had been nursed by her husband and the niece previously mentioned.

This last individual had been away from Maurice several months, having returned but a short time before. It is possible that one of these four individuals might have been a carrier. Examinations of single specimens of feces from the daughter and the cousin were negative.

It seems possible that the pleurisy and thrombophlebitis of the mother might have been the result of latent infection with typhoid bacilli, and that her illness of June was responsible for the infection of the three members of the household. We were unable to secure material from her for bacteriologic examination to decide this point.

In this outbreak of 16 cases, 4 deaths occurred. A circular giving directions for the prevention of typhoid fever and the care of the patients was distributed to each home in the town. No further cases developed.

THE ETIOLOGY OF ACUTE EPIDEMIC POLIOMYELITIS *

PLATES 1 TO 4

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The studies of the pathologic anatomy and epidemiology of acute poliomyelitis of Wickman,¹ Harbitz and Scheel,² and others, clearly indicated that the disease was infectious in nature, and the earlier work on its etiology was confined to the bacteriology of the infected tissues. These investigations yielded varying results. Early observers found micrococci of different types in poliomyelitis, but the experimental evidence brought forth as indicative of their etiologic importance is inconclusive. The first noteworthy advance towards proving the infectious nature of the disease was announced by Landsteiner and Popper³ in 1909. They reported experiments in which they had transmitted the disease to 2 monkeys by the intraperitoneal injection of a saline emulsion of the spinal cord of a child that had died during the acute stage of the disease. At about the same time Flexner and Lewis⁴ reported that they had transmitted the disease through several inoculation generations of monkeys. They also had produced the disease by various inoculation routes, and had demonstrated the infectious material in the spinal fluid, blood, nasopharyngeal mucosa, and lymph nodes near the site of inoculation.⁵ The results of these observers were confirmed by many investigators, including Knoepfelmacher,⁶ Strauss and Huntoon,⁷ Leiner and von Wiesner,⁸ and others. Furthermore Flexner and Lewis⁹ and Landsteiner and Levaditi¹⁰ found that the infectious agent was filterable through Berkefeld filters and thenceforth the etiologic agent has been spoken of as a filterable virus.

* Received for publication October 11, 1916.

¹ Die akute Poliomyelitis bzw. Heine-Medinische Krankheit, 1911.

² Pathologisch-anatomische Untersuchungen über akute Poliomyelitis und verwandte Krankheiten, 1907.

³ Ztschr. f. Immunitäts., 1909, 2, p. 377.

⁴ Jour. Am. Med. Assn., 1909, 53, pp. 1639, 1913, 2095.

⁵ Jour. Exper. Med., 1910, 12, p. 227.

⁶ Med. Klin., 1909, 5, p. 1671.

⁷ New York Med. Jour., 1910, 91, p. 64.

⁸ Wein. klin. Wchnschr., 1909, 12, p. 1698.

⁹ Jour. Am. Med. Assn., 1909, 53, p. 2095.

¹⁰ Compt. rend. Soc. de biol., 1909, 67, p. 592.

In recent years the virus of poliomyelitis has been cultivated on artificial media by Flexner and Noguchi,¹¹ using methods similar to those used in the cultivation of *Spirochaeta pallida*. In this work small pieces and emulsions of the brain and cord of monkeys dead of acute poliomyelitis were placed in tall tubes of ascites fluid containing sterile fresh rabbit kidney and the tubes incubated under anaerobic conditions. An opalescence appeared around the tissue, usually after 5 days, which increased until sedimentation occurred, about 5 days later. Examination of smears of the sediment by the Giemsa method of staining revealed the presence of small globoid bodies arranged in pairs, groups, and chains. Similar bodies were found in microscopic preparations of infected tissue and in glycerinated material. Typical lesions and death were produced in monkeys when such cultures were used for inoculation. These results led to the wide acceptance of the view that poliomyelitis is caused by a filterable virus, the bacteria that had previously been described in connection with this disease being regarded as secondary invaders.

During the summer of 1916 a severe epidemic of acute poliomyelitis prevailed in the eastern states, especially in New York, which stimulated wide-spread interest in this disease, and forcibly impressed the fact that our knowledge of its etiology and transmission was still incomplete. In Chicago a considerable number of cases occurred, from which the material for this study was obtained. In the light of accepted facts the most important problems in the etiology and transmission of acute poliomyelitis seemed to center in simplification of the methods of cultivation and identification of the infectious agent. Since it had been proved that the virus of this disease is present in the tissues of the central nervous system, fresh postmortem material was used for study; however, the blood and spinal fluid during life were studied bacteriologically in several instances. Cultures of the brain, cord, and other tissues were made on a great variety of media, some of the results of which have been announced in a previous, preliminary report.¹²

TECHNIC

The brain, spinal cord, and other material from persons dead of acute poliomyelitis were obtained under sterile conditions as soon after death as possible and cultures made immediately. Small pieces of nervous tissue were washed in sterile normal salt solution, crushed, and planted in ascites-fluid media, and ascites dextrose agar containing pieces of fresh sterile rabbit

¹¹ Jour. Exper. Med., 1913, 18, p. 461.

¹² Mathers, Jour. Am. Med. Assn., 1916, 67, p. 1019.

kidney, coagulated normal horse serum, and ascites dextrose broth. The cultures were incubated at 35-37 C. under both aerobic and anaerobic conditions for from 1 to 10 days. Usually growth appeared in some of the cultures in from 1 to 5 days. In the preparation of filtrates of the brains and cords sterile normal salt solution was used as a menstruum. The tissue suspensions were filtered through Berkefeld "N" filters and porcelain filters of the Maassen type. Cultures were made of the filtrates in the same media as the cultures from the tissues. The sterility of the media and of the rabbit tissues used was determined with special care in all cases. The characteristics, filterability, and pathogenic powers of the bacteria isolated from the materials studied were determined according to the usual methods.

In the animal experiments young rabbits, guinea-pigs, and monkeys were used.

For microscopic work the tissues were fixed in Zenker's fluid or 10% formalin, and sections stained with hematoxylin and eosin, methylene blue and eosin, and by the Gram-Weigert and Giemsa methods.

Pieces of brain and spinal cord from some of the cases were stored in 50% glycerin in the refrigerator for future use.

Cultures were made of the blood and cerebrospinal fluid before death, and of the brain, spinal cord, spinal fluid, blood, liver, kidneys, spleen, and mesenteric lymph glands after death. In 4 cases the blood cultures and in 7 cases the cultures of the cerebrospinal fluid made before death all remained sterile. In all of these cases the material was obtained early in the course of the disease, and in some of them repeated examinations of the cerebrospinal fluid were made. Of the 10 cases of poliomyelitis studied after death 4 were from Durand Hospital of the Memorial Institute for Infectious Diseases. For the rest of the material I am indebted to Dr. John Nuzum, pathologist to Cook County Hospital, Dr. E. K. Armstrong of the health department of the city of Chicago, and Dr. E. R. LeCount, physician to the coroner of Cook County.

RESULTS OF CULTURES AND EXPERIMENTS

In 9 of the 10 cases of poliomyelitis a peculiar polymorphic streptococcus-like organism was obtained from the tissues of the central nervous system in pure or mixed culture. In 2 instances staphylococci and in 1 instance *B. subtilis* were associated with this organism in the brain cultures. In 5 cases the cerebrospinal fluid was examined after death and the organism isolated in pure culture in every instance. In 2 cases the liver, kidneys, spleen, and lymph nodes were cultured and this micrococcus was isolated from the mesenteric lymph nodes and kidneys in 1 case. Cultures of the Berkefeld filtrates of brain tissue from 7 cases of poliomyelitis gave the organism in 3 instances. The record of a typical case will exemplify the method of procedure and the results of this work.

Case 5.—A man, 24 years of age, entered Durand Hospital of the Memorial Institute for Infectious Diseases, Sept. 1, 1916, with what seemed like respiratory paralysis. His sickness had begun Aug. 27, 1916, with headache and general malaise. He gradually had become worse and had gone to bed August 29.

at which time fever, nausea, and vomiting were the important symptoms. On August 30, a marked weakness of the lower extremities was noticed, which gradually increased, and when he entered Washington Boulevard Hospital on August 31, he had a flaccid paralysis of both lower extremities and a weakness of the respiratory muscles. The next day the right arm and neck muscles became paralyzed. He was then transferred to Durand Hospital. The remainder of the history is unimportant for the present purpose.

The physical examination was incomplete because of the necessity for artificial respiration. Skin cold and cyanotic. Paralysis of the muscles of respiration, of both lower extremities, and of the trunk muscles. Throat, nose, and ears normal. Submaxillary glands palpable. No rigidity of the neck. Temperature 103 F. per rectum; pulse rapid, weak, and irregular. Leukocytes 15,600, 60% polymorphonuclear, 27% small mononuclear, and 12% large mononuclear. No spinal puncture made. Death September 2.

Postmortem examination was made 1 hour after death. Marked hyperemia of meninges, brain, and cord; edema of brain and cord; hyperemia of lungs, liver, kidneys, and spleen; moderate hyperplasia of mesenteric and peribronchial lymph glands; numerous small submucous hemorrhages into the stomach; calcareous tuberculosis of right and left apices; caries of the teeth.

Brain very friable, and pinkish red in color. Cerebrospinal fluid clear but increased in amount. Cord markedly hyperemic, especially in the gray substance.

Microscopically there were numerous small hemorrhages and marked round-cell perivascular infiltration into the cord, especially the gray substance; neurophagocytosis; hyperemia and edema of cord, brain, and meninges; and small gram-positive micrococci in the gray substance of the cord (Fig. 7).

Cultures of the brain, spinal cord, and spinal fluid were made September 2 in tissue ascites fluid, ascites-fluid tissue agar, ascites dextrose broth, and coagulated normal horse serum, and incubated at 35 C. under aerobic and anaerobic conditions. Bacterial growth was apparent in all the tubes, except the ascites-fluid tissue medium, after 24 hours, and a small gram-positive polymorphous micrococcus was found in pure form. After 48 hours there was a faint turbidity around the tissue in the anaerobic ascites-fluid tissue medium, which became well developed after 72 hours. In these cultures a very small gram-positive micrococcus was found in pure form. Transfers from this culture to ascites dextrose broth yielded a growth similar to that from the brain and cord in the original ascites-dextrose-broth cultures.

On September 4, a series of 6 young rabbits was injected intravenously with the washed sediment of from 6 to 8 c.c. of the original ascites-dextrose-broth cultures of the spinal cord.

Rabbit 1.—Three days after the inoculation there was a flaccid paralysis of the right foreleg. On the next day the right hindleg was paralyzed and the animal could not walk. Killed September 8. Marked hyperemia of meninges, hyperemia with edema of brain and cord, especially of the gray matter of the cord, small subpleural hemorrhages into the cervical region of the cord; a few small subpleural hemorrhages; and hyperemia of kidneys and liver. Cocci similar in growth and form to the organisms injected were isolated from brain, cord, spinal fluid, and kidneys, but not from joints or heart blood. Microscopically, there were numerous small hemorrhages with round-cell perivascular infiltration, edema, and hyperemia in the gray matter of the spinal cord (Figs. 10, 11, 12, and 13); hemorrhages beneath the pia of brain and cord; and numerous cocci in the gray substance of the cord.

Rabbit 2.—Died 48 hours after inoculation without any evidences of paralysis. No gross changes. Cultures from brain, cord, cerebrospinal fluid, heart blood, and kidneys yielded cocci like those injected, in pure growth; joints sterile. Microscopically, there were numerous subpial hemorrhages; edema, hyperemia, and hemorrhages into the gray substance of the spinal cord, and cocci in the region of these hemorrhages.

Rabbit 3.—Three days after inoculation a flaccid paralysis of the right hind-leg developed. Killed on the 4th day. No gross changes other than marked hyperemia and edema of brain and cord and a small subpial hemorrhage in the lumbar region of the spinal cord. Cultures from brain and cord yielded the coccus injected, in pure growth; heart blood and joints sterile. Microscopically, there were small hemorrhages, edema, and round-cell infiltration into the gray substance of the cord; hemorrhages beneath the pia; and cocci in the region of the lesions of the cord.

Rabbit 4.—After 3 days flaccid paralysis of the right foreleg and on the 4th day, of the left foreleg and neck muscles, so that the animal could not move around. Killed. Marked hyperemia and edema of brain, cord, and meninges, and a few small submucous hemorrhages into the stomach. The small coccus was recovered from brain, cord, and cerebrospinal fluid in pure growth. Microscopically, there were hyperemia, hemorrhages, edema, and round-cell infiltration, especially in the gray substance of the cord, and hemorrhages beneath the pia of brain and cord.

Rabbits 5 and 6.—Died 24 hours after inoculation. No signs of paralysis before death. The coccus injected was recovered from the brain in Rabbit 5 and from the blood only, in Rabbit 6. No anatomic changes.

On September 11, a second series of 12 rabbits was inoculated intravenously with 24-hour ascites-dextrose-broth cultures of this organism. These cultures were made from single colonies on blood-agar plates, and the dose was the growth from 5-8 c.c. of a 24-hour ascites-dextrose-broth culture.

Seven of these animals developed flaccid paralysis of one or more extremities in from 4 to 7 days. One died 2 days after the inoculation; 5 died 4 days after the injection, only 1 of which showed paralysis. All of those living after 4 days developed paralysis in some form. In all of the 12 animals, characteristic lesions of the central nervous system were found. Grossly the changes consisted of marked hyperemia, edema of brain, spinal cord, and meninges. Microscopically, there were almost uniformly hemorrhages, round-cell infiltration, edema, and cellular degeneration especially in the gray substance of the spinal cord. In none of these animals were lesions of the joints noted, or bacteria recovered from the joints by cultural methods.

To summarize: 18 rabbits were inoculated with cultures of a streptococcus-like organism obtained from the brain and cord of a fatal case of poliomyelitis and 10 of these animals developed definite paralysis of one or more groups of muscles in from 3 to 7 days. In 15 animals marked changes, both gross and microscopic, were found which were similar to those in acute poliomyelitis in man.

In all the cases studied similar results were obtained. A polymorphic streptococcus-like organism was isolated, which, when injected into rabbits, produced acute inflammation of the central nervous sys-

tem like that in poliomyelitis in man. Similar results have been reported by Rosenow, Towne, and Wheeler¹³ and Nuzum and Herzog.¹⁴

CHARACTERISTICS OF THE ORGANISM ISOLATED

The micrococci isolated from the brains, cords, and spinal fluids of 9 cases of poliomyelitis were in most instances similar in form and culture. On standard human-blood-agar plates (1 part of human blood to 9 parts of plain agar) the organism grew slowly, especially early after isolation. The colonies were usually somewhat dry, small, adherent, and surrounded by faint halos of greenish discoloration. After 2 or 3 days the halo became a hazy ring of hemolysis. The colonies differ greatly from those of ordinary *Streptococcus viridans*, *pneumococcus*, and *Streptococcus pyogenes* on blood-agar plates, but the differences are perhaps not characteristic enough for ready differentiation. In 2 instances hemolysis was noticeable in the 24-hour cultures, and in 1 instance the growth on blood agar did not appear until the 3rd day of incubation. As the cultures grew more luxuriantly on continued cultivation, the hemolytic property increased.

In ascites dextrose broth the growth of all the strains was moderate. After 18 hours there was a diffuse turbidity, which rapidly became granular with a white sediment at the side and bottom of the tube. In most instances the fluid media remained turbid. Variations in oxygen tension did not materially alter the growth of these organisms. In the anaerobic ascites-fluid tissue medium the fluid around the tissue became opalescent after from 24 to 48 hours and a fine sediment collected around the tissue, gradually a finely granular haze extended upwards, and last the whole column of fluid became turbid. As a rule, the growth on the ascites-fluid tissue medium was similar to the growth of poliomyelitis virus as described by Flexner and Noguchi.¹¹ Occasionally the whole column of fluid became turbid after from 24 to 48 hours and the culture resembled in some ways the aerobic ascites-dextrose-broth cultures, but usually the anaerobic cultures required from 3 to 7 days for a good growth to develop. Two strains decolorized litmus milk and liquefied gelatin. Five strains fermented inulin. None were autolyzed in bile or normal salt solution, or agglutinated by antiserum for Groups I and II of the pneumococcus.

In form, all the strains revealed similar variations. On blood agar the organisms grew as medium-sized, gram-positive, slightly oblong

¹³ Jour. Am. Med. Assn., 1916, 67, p. 1202.

¹⁴ Ibid., 1916, 67, 1205.

micrococci in pairs or short chains. In ascites dextrose broth the form was rather variable. Some were very large round or oval diplococci (Fig. 1); others medium-sized; but usually in these cultures there were variations from minute, hardly visible, bodies to large round cocci. Often chains were seen containing both extremes in size.

The cocci usually grew in pairs, short chains, or groups, and in some ascites-dextrose-broth cultures they closely resembled staphylococci. Involution forms developed in the cultures after from 7 to 14 days, both large and small forms being seen. But these involution forms tended to correspond and a characteristic change usually took place in from 10 to 14 days when the organisms became large and stained irregularly. Large cocci with clear round or oval central areas surrounded by blue-staining borders were rather numerous, and increased in number with the age of the culture (Fig. 8). These forms disappeared on inoculation into fresh media.

In the ascites-fluid tissue medium the organisms were usually very minute, ranging from 0.25 to 0.5 microns in diameter. They were gram-positive, and arranged in pairs, chains, and groups (Fig. 3), the large forms being not uncommonly intermingled with the minute forms. In this medium the organisms also showed variable involution forms as the culture became older, and the peculiar, irregularly staining bodies just described also appeared in these cultures after from 2 to 3 weeks. The tendency of the organisms was to grow in small forms in the anaerobic ascites-fluid tissue medium and in large forms in aerobic ascites-dextrose-broth cultures, but many deviations from this tendency were noted.

Cultures of this organism passed through Berkefeld filters of the "N" type in most instances, but were removed from the media by Maassen porcelain filters. The ascites-fluid tissue medium was most favorable for the growth of the filterable form of the organism, but the other culture media were not entirely unfavorable, especially ascites dextrose broth.

The organism was usually killed by a temperature of 56 C. for from 30 minutes to 1 hour, but was resistant to glycerin. A typical culture was obtained from each of 2 human poliomyelitis brains 3 months after they had been placed in 50% glycerin.

All the strains seemed not strongly virulent for rabbits, the sediment of about 5 c.c. of an ascites-dextrose-broth culture being required

TABLE 1
MORPHOLOGIC AND CULTURAL CHARACTERISTICS OF THE ORGANISM ISOLATED FROM THE CENTRAL
NERVOUS SYSTEM IN ACUTE POLIOMYELITIS

Strain	Hemolysis	Ascites Dextrose Broth	Morphology	Litmus Milk	Plain Broth	Man- nite
1	Small dry colonies, faint green halo, no hemolysis	Cloudy sediment	No chains, large organisms	0	—	±
3	Dry colonies, faint green halo, no hemolysis	Cloudy sediment	No chains, very large forms	0	—	±
4	Dry colonies, faint green halo, no hemolysis	Cloudy sediment	No chains, very large forms	0	—	±
5	More moist than No. 3, otherwise the same	Cloudy sediment	No chains, very large forms	0	—	±
6	Same as No. 1.....	Cloudy sediment	No chains, very large forms	0	—	±
7	Very small dry colonies, faint green halo	Cloudy sediment	No chains, very large forms	No growth	—	—
8	Small dry colonies, green halo, faint hemolysis	Cloudy sediment	Chains of 20-40 cocci, very small forms	Coagulated, discolored	—	+
9	Like 8, but with greener halo	Cloudy sediment	Chains of 10-20 cocci, very small forms	Coagulated, slightly acid	—	+
10	Small dry colonies, green halo	Cloudy sediment	Pairs and short chains, medium-sized forms	0	—	—
11*	Small dry colonies, faint green halo, hemolysis after 48 hours	Cloudy sediment	Pairs, chains, large and small forms	0	—	—

* Strain 11 was isolated from the cerebrospinal fluid obtained at autopsy from a person who had died suddenly. The pathologic changes in the brain and cord were those of acute poliomyelitis.

to cause death in from 3 to 10 days. The virulence also decreased with growth on artificial media.

In describing the poliomyelitis coccus the marked tendency to variations in form is noteworthy, especially as shown on various kinds of media. Pleomorphism, however, seems to be only a minor characteristic, representing a reaction on the part of the organism to environment. In cultures of ordinary hemolytic streptococci on almost all media just as great variations in form can be observed. Staphylococci, too, when grown on various kinds of media, appear in large and in minute forms. Streptococci and staphylococci grown under anaerobic

TABLE 1—(Continued)

MORPHOLOGIC AND CULTURAL CHARACTERISTICS OF THE ORGANISM ISOLATED FROM THE CENTRAL NERVOUS SYSTEM IN ACUTE POLIOMYELITIS

Mal-tose	Dex-trose	Lac-tose	Starch	Raffi-nose	Salicin	Inulin	Solubility in Bile	Gelatin at 20 C.	Agglutination Test with Pneumococcus Sera	Filterability (Berkefeld N)
+	+	+	—	+	+	+	—	—	—	+
+	+	+	—	+	+	+	—	—	—	+
+	+	+	—	+	+	+	—	—	—	—
+	+	+	—	+	+	+	—	—	—	+
+	+	+	—	+	+	+	Dissolved	—	—	—
+	+	+	—	—	—	—	—	—	—	—
+	+	+	—	—	+	—	—	Liquefied	—	+
±	+	+	—	—	+	—	—	Liquefied	—	+
+	+	+	—	+	—	—	—	—	—	+
+	+	+	—	—	—	—	—	—	—	—

conditions in the ascites-fluid tissue medium over a period of from 3 to 7 days often produce changes in the medium and develop forms which resemble very closely those of the virus as described by Flexner and Noguchi. It may be said, however, that the tendency to develop peculiar forms is probably greater in the poliomyelitis coccus than in the other micrococci with which it might be confused. Again, this poliomyelitis coccus seems to produce lesions in the central nervous system, at least of rabbits, which is not the case with pneumococci, streptococci, or staphylococci as ordinarily seen.

The morphologic and cultural characteristics of the different strains studied in this work are shown in Table 1.

THE CHARACTER OF THE LESIONS PRODUCED IN RABBITS

The lesions of the central nervous system in animals, especially rabbits after intravenous, intraperitoneal, and intracerebral injection of cultures of the poliomyelitis coccus, were comparatively regular and characteristic. All methods of inoculation were successful, but the intravenous and intracerebral methods gave the most uniform results. The animals usually began to show symptoms of nervous lesions in from 3 to 7 days, but occasionally only after a much longer time. In one instance 3 weeks elapsed before the rabbit developed paralysis. The manifestations in rabbits were varied; special care was exercised to determine whether paralysis actually was present or some other condition, as arthritis, myositis, or the profound general weakness which often precedes death in these animals. In most instances the picture was striking. In from 3 to 7 days after inoculation the animal would walk with some difficulty and rapidly a flaccid paralysis of one or more limbs would develop, the general condition, however, often being still good. Paralysis of the muscles of one or more extremities, of the neck, and of the respiratory muscles was seen repeatedly. A few died early after the paralysis, sometimes in convulsions, and sometimes suddenly, with rapidly developing respiratory embarrassment.

The lesions in the rabbit were similar to those described as characteristic of acute poliomyelitis in man. Edema and hyperemia of brain and cord associated with subpial hemorrhages were almost constantly found. The brains of rabbits with nervous symptoms were always larger than normal, very moist, friable, deep-pink or red in color, with small subpial hemorrhages especially in the region of the medulla. Occasionally a large hemorrhage beneath the pia was found. The cerebrospinal fluid was turbid, increased in amount, and under pressure. And in these animals the spinal cord was swollen, moist, and often so soft that it could not be removed intact. The spinal canal was usually filled completely by the cord. On section the gray matter would often stand out as a pink area.

Microscopically, the lesions were also striking. Small subpial hemorrhages (Fig. 9) were constant, especially in the fissures of the brain and the central fissure of the cord. Hemorrhages into the gray substance (Figs. 10 and 11) of varying degrees and round-cell infiltration especially around the blood vessels (Figs. 12 and 13), with edema and hyperemia were the most noticeable changes in the cord. Neurophagocytosis, diffuse round-cell infiltration, and degeneration were frequently

present. Micrococci were demonstrated regularly in the lesions both of man (Figs. 4 and 7) and of animal (Figs. 5 and 6).

Sterile filtrates of brains of poliomyelitis patients were injected intraperitoneally into guinea-pigs and intracerebrally, intraperitoneally, and intravenously into rabbits. Doses of from 10 to 25 c.c. of 100% filtrates were used for both rabbits and guinea-pigs. In no instance did the guinea-pigs or rabbits thus inoculated show any evidences of infection.

Because of the scarcity of monkeys only a few experiments on these animals have thus far been made. Four monkeys (*M. rhesus*) have been inoculated with freshly isolated cultures of the poliomyelitis coccus. One received the bacteria in 15 c.c. of a 24-hour ascites-dextrose-broth culture intravenously, another a similar dose intraperitoneally, a third the growth from 0.5 c.c., and a fourth that of 1 c.c. of an ascites-dextrose-broth-culture intracerebrally. The one injected intravenously seemed ill for several days, but recovered without any manifestations of paralysis. The monkey injected intracerebrally with the organisms in 1 c.c. of a 24-hour ascites-dextrose-broth culture of a strain of the poliomyelitis coccus became ill 4 days later, and developed flaccid paralysis of the left arm. After an illness of about 2 weeks, improvement set in, and partial use of the paralyzed arm has been regained. The other two monkeys remained normal following inoculation with this organism.

Experiments bearing on the true relation of this polymorphic coccus to the virus of poliomyelitis described by Flexner and Noguchi and immunologic studies will be included in subsequent publications.

SUMMARY

In a bacteriologic examination of fresh material from 10 cases of acute poliomyelitis a peculiar polymorphic streptococcus-like organism has been isolated in 9 instances, in 7 of which the growth has been pure. Similar organisms have been demonstrated microscopically in the tissues of the central nervous system of these cases. Cultures of this coccus injected into rabbits, have produced paralysis of various groups of muscles, and characteristic lesions in the central nervous system consisting of hyperemia and edema of the tissues, with hemorrhages, round-cell perivascular infiltration, and neurophagocytosis in the spinal cord, especially in the gray substance, similar in every detail to the changes considered characteristic of acute poliomyelitis in man.

This micrococcus has been recovered from the lesions in the inoculated rabbits by both cultural and microscopic methods.

The artificial cultivation of the poliomyelitis coccus in an ascites-fluid tissue medium under anaerobic conditions causes changes in the media which cannot be differentiated from those previously described for cultures of the so-called virus of poliomyelitis. Morphologically, also, this bacterium when grown on the same media is similar to the virus, and in stained smears it appears in minute gram-positive coccus-like bodies arranged in pairs, groups, and chains. These minute forms disappear when the organism is cultivated in other media under aerobic conditions.

The morphologic, cultural, and pathogenic characters of the poliomyelitis coccus thus far determined indicate that it is an important factor in the disease.

EXPLANATION OF PLATES

PLATE 1

FIGS. 1 and 2. Large forms of the poliomyelitis coccus. Ascites-dextrose-broth culture. Gram stain. $\times 1200$.

FIG. 3. Small forms of the poliomyelitis coccus. Anaerobic ascites-fluid tissue culture. Gram stain. $\times 1200$.

FIG. 4. Microorganisms in stained smear of the fresh brain from a fatal case of poliomyelitis. Gram stain. $\times 1200$.

FIGS. 5 and 6. Microorganisms in the gray substance of the spinal cord of a paralyzed rabbit dying after injection of a culture of the poliomyelitis coccus. Gram-Weigert stain. $\times 1200$

PLATE 2

FIG. 7. Microorganisms in the gray substance of the spinal cord of a patient that died of poliomyelitis. Gram-Weigert stain. $\times 1200$.

FIG. 8. Peculiar degeneration forms of the poliomyelitis coccus. Gram stain. $\times 1200$.

FIG. 9. Subpial hemorrhages in the brain of a rabbit injected with the poliomyelitis coccus. Hematoxylin and eosin. $\times 60$.

PLATE 3

FIG. 10. Small hemorrhages in the gray substance of the spinal cord of a paralyzed rabbit injected with a culture of the poliomyelitis coccus. Methylene blue and eosin. $\times 110$.

FIG. 11. Same as Fig. 10. $\times 425$.

PLATE 4

FIG. 12. Round-cell infiltration in the gray substance of the spinal cord of a rabbit that died after injection of a culture of the poliomyelitis coccus. Hematoxylin and eosin. $\times 150$.

FIG. 13. Same as Fig. 12. $\times 425$.

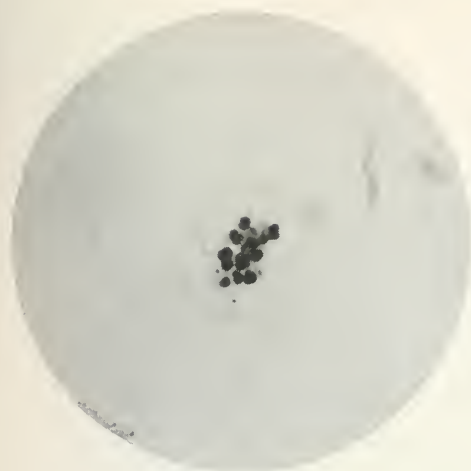


Figure 1

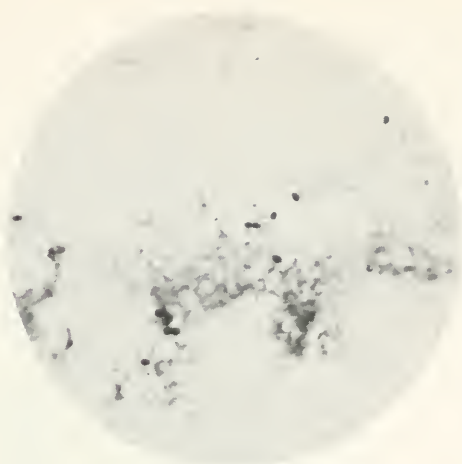


Figure 4



Figure 2



Figure 5



Figure 3



Figure 6

PLATE 2



Figure 7

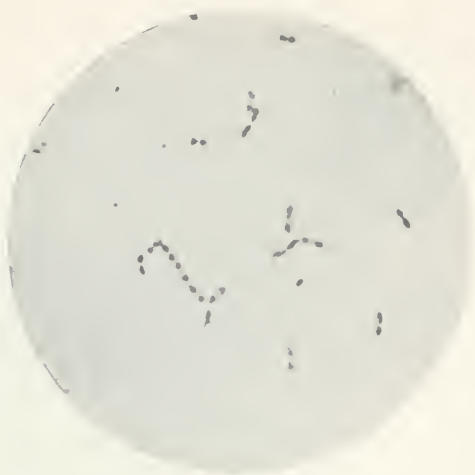


Figure 8



Figure 9

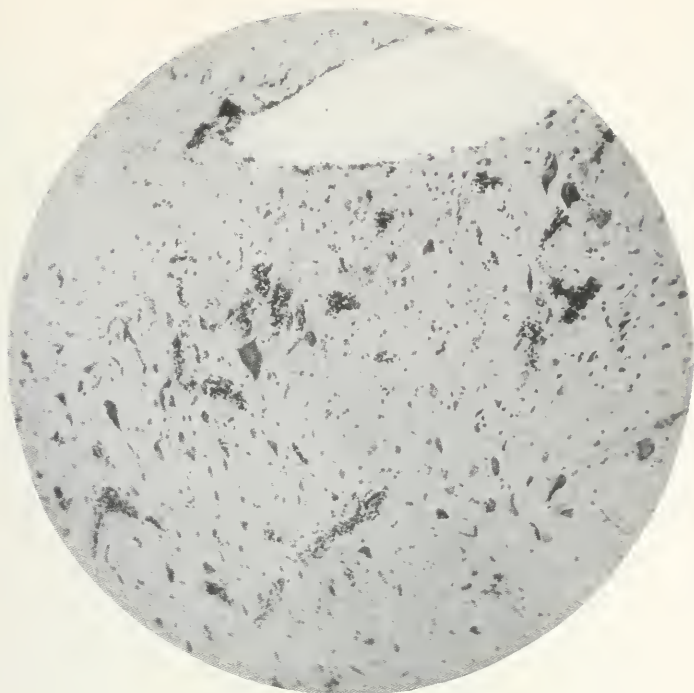


Figure 10

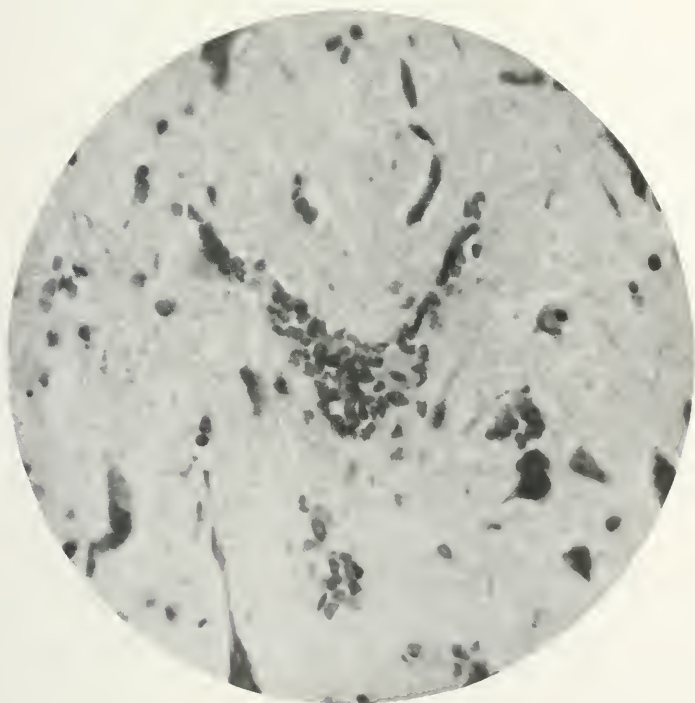


Figure 11



Figure 12

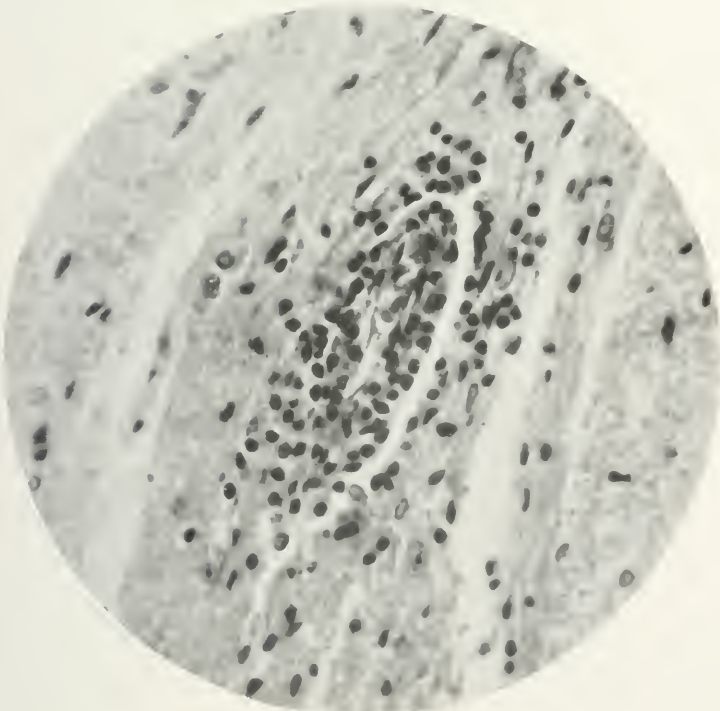


Figure 13

THE VIRULENCE OF DIPHTHERIA BACILLI FROM DIPHTHERIA PATIENTS AND DIPHTHERIA- CARRIERS *

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The study here reported began in the testing for virulence of cultures from diphtheria-bacillus-carriers as they were met in Durand Hospital. It was extended to include cultures from diphtheria patients taken early in the disease in order that comparison might be made with later cultures if the bacilli should persist unduly long, and also cultures from diphtheria patients who had harbored the bacilli so long that they might properly be classed as carriers. The carriers were admitted as such or were nurses on duty in the hospital. They did not give histories of diphtheria and had been detected as carriers in routine examinations. In some of these it was impossible to exclude very mild attacks of diphtheria at times more or less remote from the moment at which they came under observation. It is probable that nearly all of them were contacts, tho this could not be determined in regard to most of them from the facts available. The carriers among the nurses were detected in routine examination of the throat and nose. Negative Schick reactions showed all of them to be immune at the time, either naturally or as the result of the administration of diphtheria antitoxin. In fact, all the carriers gave negative Schick reactions with the possible exception of two encountered early in the study on whom this test was not made. My purpose was to determine what proportion of the bacilli which are recognized as diphtheria bacilli in the routine work are virulent. I made no effort to measure the degree of virulence of the individual cultures.

TECHNIC

Cultures from the throat and nose on Loeffler's serum medium were incubated about 20 hours. From these cultures, by repeated plating, pure cultures were secured and studied as to their morphology and cultural peculiarities and tested for their pathogenic properties in guinea-pigs. Usually but one pure culture was tested in each instance.

All the cultures were of the granular or barred types, corresponding with Types A, A', C, C', and D of Wesbrook, Wilson, and McDaniel.¹ In the

* Received for publication November 8, 1916.

¹ Rep. Minnesota State Bd. of Health and Vital Statistics, 1901, p. 613.

cultures usually a few and often very numerous short forms were associated with the predominating longer and medium-length ones. Most of the cultures after 20 hours on Loeffler's medium showed abundant granules with Neisser's stain, but this was inconstant, no granules being observed in 3 typical virulent strains.

In litmus milk all the cultures produced a faint reddening, and in dextrose litmus broth (Smith's sugar-free broth 1% dextrose) acid was produced by all save one. The last grew scarcely at all in this medium, but when ascites fluid was added, it grew well and produced acid. In deep stab cultures in dextrose agar all grew to the bottom of the puncture and none spread over the surface.

In testing the virulence of cultures, a 24-hour growth on a slant of Loeffler's medium was used. The cutaneous test was employed for 55 cultures, the subcutaneous test for 35 cultures, and both tests for 26 cultures. When both tests were used, the results corresponded.

In the cutaneous tests 2 guinea-pigs were used, one serving as a control. The control received 50 units of diphtheria antitoxin intraperitoneally 24 hours before inoculation with the cultures. Both guinea-pigs were inoculated intracutaneously with a sharp, flat-pointed needle, slightly bent near the end after the method of Riemsdijk.² Four or 6 cultures were usually tested on 1 guinea-pig. A culture was classed as virulent if a local reaction appeared in the test animal after 24 hours while the control showed none.

In subcutaneous tests the control received 25 units of diphtheria antitoxin intraperitoneally 24 hours before inoculation. Both the control and test animals received from one-eighth to one-fourth of the 24-hour growth on the surface of a slant of Loeffler's medium subcutaneously in the abdominal wall. The culture was classed as virulent if the control remained well while the test animal died showing the typical lesions, including local exudation, edema, and hyperemic adrenals.

Corresponding tests were carried out by both methods, with typical cultures of Hofmann's bacillus and cultures resembling diphtheria bacilli morphologically, but differing from them in growth in litmus milk, dextrose broth, and dextrose agar (diphtheroid bacilli). None of these cultures was fatal for guinea-pigs, but a few after intracutaneous inoculation caused slight local reactions in both test and control animal.

All cultures from patients with diphtheria and from carriers were virulent. Nonvirulent diphtheria bacilli were not found.

The cultures came from 36 patients with typical diphtheria at varying intervals after the onset of the disease, and from 14 carriers. In 31 of the cases of diphtheria a single culture was tested. These cases are recorded in Table 1. The number of days elapsing between the time when the culture was obtained and the time when the bacilli disappeared from the throat or nose, is indicated in each case. It will be noticed that the tested culture was sometimes secured shortly before cultures became negative. In 5 cases, 2 or more cultures were studied. These are recorded in Table 2. In each case the later cultures corre-

² *Folia Microbiologica*, 1915, 4.

TABLE 1
PERSISTENCE OF VIRULENT DIPHTHERIA BACILLI IN PATIENTS WITH DIPHTHERIA

Culture No.	Source of Culture	Day of Disease on Which the Tested Culture Was Made	Number of Days Before Subsequent Cultures Became Negative
21	Throat	1st	11
10	Throat	2nd	19
13	Throat	2nd	16
24	Throat	2nd	2
41	Nose	2nd	9
47	Throat	3rd	2
69	Throat	3rd	6
23	Nose	4th	17
28	Throat	4th	20
33	Throat	4th	17
40	Throat	4th	21
39	Throat	4th	5
59	Throat	4th	19
51	Throat	4th	1
52	Throat	5th	Died
2	Throat	6th	Died
25	Throat	6th	32
31	Throat	7th	23
48	Laryngeal tube	7th	No positive cultures from throat
29	Laryngeal tube	8th	No positive cultures from throat
63	Nose	8th	26
7	Throat	11th	9
60	Nose	11th	42
49	Nose	13th	4
3	Throat	14th	17
27	Throat	14th	10
70	Throat	27th	5
42	Throat	28th	8
5	Throat	29th	1
4	Throat	34th	14
19	Throat	36th	12

TABLE 2
PERSISTENCE OF VIRULENT DIPHTHERIA BACILLI IN PATIENTS WITH DIPHTHERIA

Culture No.	Source of Culture	Day of Disease on Which the Tested Culture Was Made	Number of Days Before Subsequent Cultures Became Negative
Case 1 {	50	1	4
	57	2	3
Case 2 {	37	2	25
	44	21	6
Case 3 {	32	2	32
	35	13	21
	45	32	4
Case 4 {	15	4	39
	16	5	38
	20	38	5
Case 5 {	53	7	19
	61	25	1

sponded in every way with the earlier ones and were uniformly virulent.

There was no evidence in the results secured in the study of the diphtheria patients to indicate that the bacilli become nonvirulent after remaining a considerable time on the infected surface, even when tested shortly before their final disappearance.

The 14 carriers of diphtheria bacilli are enumerated in Table 3.

TABLE 3
PERSISTENCE OF VIRULENT DIPHTHERIA BACILLI IN DIPHTHERIA-BACILLUS-CARRIERS

Case No.	Culture No.	Source of Culture	Days After Entrance Before Tested Culture Was Made	Number of Days Before Subsequent Cultures Became Negative
1	1	Throat	7	1
2	64	Throat	1	3
3	30	Throat	1	4
4	38	Throat	1	4
5	17	Nose	6	5
6	43	Throat	2	7
7	65	Throat	1	9
8	12	Throat	1	12
9	58	Throat	1	12
10	14	Throat	1	14
11	67	Nose	2	39
12	{ 45	Nose	2	72
	{ 46	Nose	34	40
	{ 54	Nose	58	16
13	{ 66	Throat	1	53
	{ 68	Throat	47	7
14	{ 71	Nose	7	
	{ 73	Nose	84	
	{ 74	Nose	129	

Three were nurses on duty in Durand Hospital. The remaining 11 were admitted as carriers, usually having been detected as such in routine bacteriologic examinations by the Chicago health department. None of these gave a history of having had diphtheria, altho some had had slight sore throat or "cold in the head" some days or weeks previously. Three had had a purulent nasal discharge. Most of these carriers got rid of the bacilli in a short time. In 3, positive cultures were very persistent, and there was no evidence during a long period of observation that the bacilli were becoming nonvirulent.

The conclusions reached in this study agree very well with those of previous workers, with whom the general observation has been that cultures from cases of clinical diphtheria are almost always virulent.

In the virulence tests the culture to be injected should show a vigorous growth, and therefore a favorable medium should be selected. This is emphasized by the experience of Williams,³ who found that some of the segmented

³ Jour. Med. Research, 1902, 8, p. 83.

varieties of the diphtheria bacillus grew scantily in broth, and that such cultures, except in large amounts, gave little reaction in guinea-pigs, but that when grown in ascites broth, in which they grew rapidly and abundantly, they showed a high degree of toxicity. Some of my cultures grew very slowly and poorly in broth, even after repeated transfers, but grew well on Loeffler's serum medium. Satisfactory results followed the injection of young cultures on Loeffler's medium, preceded in the control animal by a relatively large dose of diphtheria antitoxin.

The prevalence of carriers of diphtheria bacilli has been the subject of much study. Graham-Smith⁴ has tabulated the results of a large number of observers on the infection of healthy contacts with diphtheria bacilli. These statistics show that of close contacts—members of infected families, relatives, and attendants—36.6% are liable to become infected, that in hospital wards and institutions 14% are liable, and among scholars of infected schools, 8.7%. In Durand Hospital biweekly cultural examinations are made of the throats of nurses on duty. During the past 2 years, of 66 nurses, 10 became carriers, or 15.15%. None of these developed diphtheria. Goldberger, Williams, and Hachtel⁵ undertook extensive examinations to determine the number of diphtheria-carriers among the general population of Detroit. Of 4093 apparently healthy persons without history of contact, 38 (0.928%) harbored morphologically diphtheria bacilli. Of 19 cultures isolated, 2 were virulent, suggesting a possible 0.097% of the individuals examined as carriers of virulent diphtheria bacilli. The remaining 17 cultures correspond with what are designated nonvirulent diphtheria bacilli.

This wide distribution of diphtheria bacilli in apparently healthy persons renders the relationship between virulent and nonvirulent strains of great practical importance, but the relationship is not entirely clear. Several times it has appeared to have been shown that nonvirulent strains of diphtheria bacilli had been secured from virulent ones, but it has not been possible to convert a nonvirulent into a virulent form. Most of the persons who have considered this matter from a practical standpoint have reached the conclusion that carriers of nonvirulent diphtheria bacilli are not a source of danger to others, and that such persons should not be subject to isolation. It has been a matter of much interest to know whether diphtheria bacilli in the throat and nose lose their virulence after prolonged residence. After reviewing the available data on this point, Graham-Smith⁴ reaches the

⁴ The Bacteriology of Diphtheria, 1908, p. 186.

⁵ Bull. 101, Hyg. Lab., Washington, D. C., p. 29.

conclusion, held by most of those who have investigated the subject, that in a few instances the bacilli appear to have lost virulence to some extent, but that in the great majority they have retained their full virulence up to the time of their final disappearance. As stated, I have been unable to discover, either in carriers or in persons who have had diphtheria, a loss in virulence on the part of the bacilli after a prolonged residence in apparently healthy noses and throats, even tho they were secured a short time before their final disappearance.

Recent observations from several sources have emphasized the fact that persistent carriage of diphtheria bacilli usually depends on some local condition, such as diseased tonsils, adenoids, or sinuses. In such cases the bacilli escape in small numbers, except periodically, when they may appear on the surface in abundance. While such cases are probably less dangerous as spreaders of infection than recent cases of diphtheria with abundance of infective secretions, still they must be considered as potential agents for dissemination of the disease and must be isolated until the bacilli have been shown to be nonvirulent.

CONCLUSIONS

Diphtheria bacilli from patients with diphtheria and from carriers who have been in contact with such patients are practically always virulent.

The bacilli in such cases usually remain virulent up to the time of disappearance, even tho a long time has elapsed.

Carriers should be kept in isolation until the bacilli have been gotten rid of, or until the strains cultivated have been proved to be non-virulent.

Cultures from the nose, as well as from the throat, should be made in all suspected cases.

THE ALLEGED DETOXICATING POWER OF THE THYROID GLAND *

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In 1902 Remedi¹ reported that the injection of extracts of killed cultures of *Bacillus prodigiosus*, *B. anthracis*, *B. diphtheriae*, and *B. tetanus* uniformly into the two lobes of the thyroid gland, brought about an increased secretion of colloid and averted, or greatly ameliorated, the symptoms of intoxication usually produced by the injection of these bacterial extracts subcutaneously.

Since the literature contains conflicting statements with regard to the action of the thyroid gland in different toxemias, I have carried out series of experiments somewhat similar to those of Remedi. Two series of experiments were undertaken. In the first series, minimal, subminimal, and repeated subminimal lethal doses of diphtheria toxin were injected uniformly into one, and into both thyroid glands of the dog. In the second series tetanus toxin was injected in the same way.

HISTORICAL REVIEW

That secretory epithelium, especially that of the thyroid gland, is remarkably sensitive to toxic bacterial products is well known, but whether this brings about increased secretory activity on the part of the thyroid gland is not so certain. Galleotti² found that toxic products of metabolism, when injected into the peritoneal cavity of the turtle, brought about a more marked secretion in the thyroid gland. von Blum³ is of the opinion that the thyroid neutralizes certain toxic substances in the blood, and he asserts that the presence of such toxic products constitutes the normal stimulation of the cells of the thyroid gland to secretory activity and hypertrophy. Roger and Garnier⁴ state that in the acute infectious diseases there is a hypersecretion with an increased amount of colloid in the thyroid gland. In the more chronic diseases, as tuberculosis and typhoid fever, they found a disappearance of colloid, associated with proliferation of the epithelium of the thyroid gland. Torri⁵ in similar studies came to the same conclusions. These results could not be confirmed by Kaschwamura.⁶

Many investigators claim, on the other hand, that the parenchymatous

* Received for publication October 17, 1916.

¹ *Sperimentale*, 1902, 56, p. 500.

² *Internat. Monatschr. f. Anat. u. Physiol.*, 1895, 12, p. 513. *Arch. f. Microscopic Anat.*, 1896, 48, p. 305.

³ *Virchows Arch. f. path. Anat.*, 1899, 158, p. 495.

⁴ *Compt. rend. Soc. de biol.*, 1898, 50, pp. 873, 889. *Presse méd.*, 1899, 7, p. 181.

⁵ *Policlinico*, 1900, n. 6, 8, 10.

⁶ *Virchows Arch. f. path. Anat.*, 1901, 166, p. 373.

changes predominate over the colloid changes. They believe that many outside influences will bring about hyperplastic changes. Marine⁷ has shown in the brook trout how easily the thyroid gland is changed from a colloid gland to a hyperplastic gland by overfeeding, by unsuitable food, by overcrowding, or by other unhygienic factors, and that the condition can be readily cured or prevented by improving and changing these conditions. McCarrison⁸ also found that inanition, unhygienic surroundings, or poor food favor a bacterial toxemia from the gastro-intestinal tract, which induces secondary, that is hyperplastic, changes in the thyroid gland. More recently Bensley⁹ showed that the spontaneous hyperplasia of the thyroid gland in the opossum can be produced and controlled by diet alone.

Farrant¹⁰ found marked changes in the thyroid glands of over 700 persons dying of various infectious diseases. He claims that the rapidity of the transformation of a normal thyroid gland into a hyperplastic gland depends not only upon the acuteness and the duration of the infection, but also upon the kind of infection. Farrant produced hyperplasia experimentally by subcutaneous injection of diphtheria toxin. Feeding desiccated thyroid gland along with injection of diphtheria toxin decreased the toxicity of the diphtheria toxin, the animals thus fed surviving, by one or two days, those that were not so fed. Emge,¹¹ on the other hand, by using lethal and sublethal doses of diphtheria toxin, could not produce any parenchymatous changes in the thyroid gland similar to those observed by Farrant. Furthermore, Emge by producing experimental diphtheria toxemia, could not induce changes in the thyroid gland similar to those observed in the human thyroid gland in cases of fatal diphtheria.

The relation of the thyroid gland to the formation of antibodies and immunity has also been studied. Fassin¹² observed a diminution in the hemolytic and the bacteriolytic alexins of the blood after thyroidectomy. Marbe¹³ concluded that hyperthyroidism, induced by feeding desiccated thyroid extract to rabbits and guinea-pigs, distinctly augmented the opsonic power of the blood serum. In a later paper Marbe reported a decrease of the opsonic and phagocytic powers of the blood in thyroidectomized dogs. Frouin¹⁴ partially confirmed Fassin's work relative to the diminution of the alexins in the blood after thyroidectomy in dogs. However, he did not notice any decrease in the amount of tetanus antitoxin in the blood serum of thyroidectomized dogs which had been immunized against tetanus toxin. Furthermore, Lerde and Diez¹⁵ found that thyroidectomized guinea-pigs are about as resistant to diphtheria and tetanus toxin as normal guinea-pigs. Fjeldstad¹⁶ did not observe any difference in the degree of immunity and the rapidity of appearance of the agglutinins for the typhoid bacillus in rabbits immunized after removal of the thyroid glands. More recently Launoy and Levy-Bruhl¹⁷ reported that the removal of the thyroid glands in chickens had no effect on the course of a spirochetal infection (*Spirochetosis gallinarum*).

⁷ Bull. Johns Hopkins Hosp., 1910, 21, p. 95.

⁸ Indian Jour. Med. Research, 1914, 2, p. 183.

⁹ Am. Jour. Anat., 1916, 19, pp. 37, 57.

¹⁰ Brit. Med. Jour., 1914, 1, p. 470. Lancet, 1913, 2, p. 1820.

¹¹ Jour. Infect. Dis., 1915, 17, p. 369.

¹² Compt. rend. Soc. de biol., 1907, 62, p. 647.

¹³ Ibid., 1908, 64, pp. 1058, 1113.

¹⁴ Ibid., 1910, 69, p. 237.

¹⁵ Gior. d. r. Accad. di med. di Torino, 1905, 11, pp. 195, 429.

¹⁶ Am. Jour. Physiol., 1910, 26, p. 72.

¹⁷ Ann. de l'Inst. Pasteur, 1915, 29, p. 213.

EXPERIMENTS WITH DIPHTHERIA TOXIN

The minimal lethal dose of diphtheria toxin (the amount required to kill a 250-gm. guinea-pig in 5 or 6 days) was found to be 0.004 c.c. This was used as a standard dilution. All dilutions were made with sterile physiologic salt solution (0.85%).

Controls.—Healthy dogs were selected for all the experiments, care being taken to exclude those with visible or palpable evidence of enlarged thyroid glands. The dogs weighed approximately 8 kilos each. It was found that the minimal lethal dose of diphtheria toxin per kilo of body weight of the dog was practically the same as that for the guinea-pig. The calculated dose was diluted with physiologic salt solution to 2.5 c.c. This was injected subcutaneously about the region of the thyroid gland. The onset of symptoms in all the controls was very much the same. The dogs refused all food on the 2nd day. Respiration and pulse rates rapidly increased. Rectal temperature was from 2 to 4 degrees above normal. Death occurred on the 4th day in 2 cases and on the 5th day in 1 case. The thyroid glands were removed and carefully weighed. Thyroid tissue was removed from the center of both the right and left of the gland for histologic examination. All sections were fixed in formalin-Zenker, imbedded in celloidin, and stained with hematoxylin and eosin. Sections taken through the center of the two glands are for practical purposes very similar in histologic structure. This fact is taken into consideration in later experiments when the two glands were compared after one of them had been injected with diphtheria or tetanus toxin. The amount and stainability of the colloid and the presence of hemorrhages and necrosis were especially noted. The type of epithelium and any evidence of active hyperplasia and mitosis were also noted. The results are given in Table 1. The thyroid glands in the control dogs had a normal amount of colloid, the amount of parenchymatous tissue was normal, and the epithelium was of the cuboidal type. No mitosis or any other sign of active hyperplasia was observed.

Minimal Lethal Doses Injected Into Both Glands.—Two dogs were weighed and prepared for operation. Ether anesthesia was used in all cases. The thyroids were exposed through the usual midline incision. The minimal lethal doses were carefully calculated and the dilutions so made that the entire amount injected into both thyroid glands would not be more than 2.5 c.c. The calculated dose was injected as diffusely as possible with the least possible traumatism. The glands were immediately put back in place and the wound closed.

The onset of symptoms was more pronounced and rapid than in the controls. Death occurred from 1 to 2 days sooner than in those injected subcutaneously. Autopsy revealed the usual hemorrhage in the gastro-intestinal tract. The wound about the thyroid gland was healing and there was no evidence of any infection. There was some edema about the thyroid glands but no hemorrhage. The glands were congested, edematous, and had a swollen appearance, but were approximately equal in weight (see Table 1). Microscopic examination: Alveoli of normal size. Colloid stained uniformly. No evidences of an increased formation of colloid. No necrosis. Desquamation of epithelial cells from the walls of the alveoli was most marked in the thyroid gland of Dog 5.

Subminimal Lethal Doses Injected Into Left Gland.—Subminimal lethal doses (one-tenth of the minimal lethal dose) were injected as diffusely as possible into the left thyroid gland. The dogs developed only slight passing symptoms of toxemia. Dogs 3 and 7 were killed with ether on the 7th and 10th

TABLE 1
RESULTS ON THYROID GLAND ACTIVITY OF INJECTION OF DIPHTHERIA TOXIN

Dog	Weight (kilos)	Amount Injected (c.c.)	Site of Injection	Repeated Injections	Date of Death
1	7.9	2.5 = M.L.D.	Subcutaneously about thyroid gland		4th day
2	7.8	2.5 = M.L.D.	Subcutaneously about thyroid gland		5th day
3	8.9	2.9 = M.L.D.	Subcutaneously about thyroid gland		4th day
4	6.2	1.5 = M.L.D.	Both thyroid glands, diffusely		3rd day
5	6.9	1.9 = M.L.D.	Both thyroid glands, diffusely		3rd day
6	7.8	1.9 = 1/10 M.L.D.	Left thyroid gland, diffusely		Killed, 4th day, with ether
7	8.5	1.2 = 1/10 M.L.D.	Left thyroid gland, diffusely		Killed, 7th day
8	8.0	1 = 1/10 M.L.D.	Left thyroid gland, diffusely	Repeated in 15 days	Killed, 21st day
9	7.5	1.5 = 1/10 M.L.D.	Left thyroid gland, diffusely	Repeated in 15 days	Killed, 21st day, with ether
10	8.5	1.8 = 1/10 M.L.D.	Left thyroid gland, diffusely	Repeated in 15 and 30 days	Killed after 6 weeks
11	9.5	2.5 = 1/10 M.L.D.	Left thyroid gland, diffusely	Repeated in 15 and 30 days	Killed after 6 weeks
12	8.6	1.8 = 1/10 M.L.D.	Left thyroid gland, diffusely	Repeated in 15 and 30 days	Killed after 6 weeks
13	9.4	2 = 1/10 M.L.D.	Left thyroid gland, diffusely	Repeated in 15 and 30 days	Killed after 6 weeks
14	7.5	1.5 = 1/10 M.L.D.	Both thyroid glands, diffusely	Six repeated injections	Killed after 3 months
15	8.4	1.8 = 1/10 M.L.D.	Both thyroid glands, diffusely	Six repeated injections	Killed after 3 months
16	8.0	10 = M.L.D.	Liver, diffusely		2nd day
17	8.5	10 = M.L.D.	Spleen, diffusely		2nd day
18	9.5	3 = M.L.D.	Testicle, diffusely		4th day

days respectively. The left glands were increased in weight (see Table 1), edematous and hemorrhagic. Microscopic examination: Colloid in the injected glands was the same in amount as in the normal glands, stained uniformly, and did not distend the alveoli. Few small foci of hemorrhages found. Epithelium of the cuboidal type. Some desquamation of the epithelial cells from the walls of the alveoli. No evidence of any recent active hyperplasia of the epithelial cells. No mitoses.

Repeated Subminimal Lethal Doses Injected Into Left Gland.—Repeated subminimal lethal doses (one-tenth of the minimal lethal dose) of diphtheria toxin were injected as diffusely as possible into the left thyroid. In Dogs 8 and 9 the injections were repeated in 15 days. One of the dogs was killed with ether 21 days after the first injection and the other 30 days after the first injection. The injected thyroid glands showed an increase in weight (see Table 1). Microscopic examination: Marked hemorrhage, edema, and desquamation of epithelium in the injected glands. Less colloid than in the right glands, which were not injected. The colloid did not stain uniformly in the glands that were injected. No evidence of any beginning of active hyperplasia of epithelium. No mitosis of epithelium.

In Dogs 10, 11, 12, and 13 the injections were repeated in 15 and 30 days, and the dogs killed in 6 weeks after the initial injection. The increase in weight

TABLE 1 (Continued)
RESULTS ON THYROID GLAND ACTIVITY OF INJECTION OF DIPHTHERIA TOXIN

Weight of Thyroid Gland		Histologic Description of Thyroid Glands
Right (gm.)	Left (gm.)	
3.6	3.5	Colloid type. Normal
1.2	3.9	Colloid type. Normal
8.5	8.0	Colloid-moderate hyperplastic (reverting) type. Normal
7.6	7.0	Colloid type. Some hemorrhage. No mitosis. No increase in colloid
4.6	5.0	Hyperplastic type. Little colloid. Edema. Hemorrhage. Desquamation
4.2	5.4	Hyperplastic type. Little colloid. Left: hemorrhage. Little colloid. Some desquamation of epithelium
6.2	8.8	Colloid-moderate hyperplastic type. Left: hemorrhages. No other changes
4.6	4.8	Colloid-moderate hyperplastic type. Left: hemorrhage. Edema. Desquamation of epithelium. Less colloid
8.5	10.8	Colloid-moderate hyperplastic type. Left: desquamation of epithelium. Less colloid. Small blood vessels
5.0	4.8	Colloid type. Left: no colloid. Desquamation of epithelium. Many small blood vessels
3.8	5.2	Colloid-slight hyperplastic type. Left: no colloid. No increase but desquamation of epithelium. Small blood vessels
4.6	4.9	Colloid-moderate hyperplastic type. Left: no colloid. Desquamation of epithelium
3.5	3.3	Colloid-moderate hyperplastic type. Left: less colloid. Desquamation. Hemorrhage
4.2	4.0	Colloid-moderate hyperplastic type. Little colloid. Desquamation of epithelium
9.8	9.6	Hyperplastic type. Little colloid. Marked desquamation
6.2	6.7	Colloid type. No changes
9.4	11.6	Colloid-moderate hyperplastic type. No changes. Some desquamation of epithelium
8.6	8.5	Colloid-slight hyperplastic type. Some desquamation of epithelium

of the injected glands (left) over the glands which were not injected is not so marked in these cases. In 2 only, was there any increase in weight. Microscopic examination: Marked decrease of colloid. Colloid did not take the stain uniformly. Many of the alveoli empty and shrunken. Desquamated epithelial cells scattered about the empty alveoli. No evidence of any recent active hyperplasia of epithelium. No mitosis.

In Dogs 14 and 15, subminimal lethal doses of diphtheria toxin were injected 6 times at intervals of 2 weeks. Both dogs were killed with ether 10 days after the last injection, and the thyroid glands were removed for histologic examination. There was no evidence of any infection about the thyroid glands. Microscopic examination: Marked decrease in the amount of colloid in all the sections. Many of the alveoli were empty, and the colloid that was still left in the alveoli did not take the stain uniformly. Desquamation of epithelium from the walls of the alveoli marked, but no evidence of any active hyperplasia on the part of the epithelial cells. No mitosis. In the thyroid glands of Dog 15 there was practically no colloid. The epithelium of many of the alveoli had entirely desquamated, and these cells were scattered about the empty alveoli. No evidence of any active hyperplasia. No mitosis.

Minimal Lethal Dose Injected Into Liver, Spleen, and Testicles.—Minimal lethal doses of diphtheria toxin were injected diffusely into liver, spleen, and

TABLE 2
RESULTS ON THYROID GLAND ACTIVITY OF INJECTION OF TETANUS TOXIN

Dog	Weight (kilos)	Amount Injected (c.c.)	Site of Injection	Repeated Injections	Date of Death
1	8.5	2.5 = M.L.D.	Subcutaneously about thyroid gland		5th day
2	7.4	2.5 = M.L.D.	Subcutaneously about thyroid gland		5th day
3	9.0	2.5 = M.L.D.	Subcutaneously about thyroid gland		4th day
4	8.0	2.5 = M.L.D.	Both thyroid glands, diffusely		6th day
5	7.8	2.5 = M.L.D.	Both thyroid glands, diffusely		5th day
6	8.5	1.2 = M.L.D.	Left thyroid gland, diffusely		6th day
7	9.0	2.5 = 1/10 M.L.D.	Both thyroid glands, diffusely		Killed, 20th day
8	8.4	1.5 = 1/10 M.L.D.	Left thyroid gland, diffusely	Repeated in 15 days	Killed, 20th day
9	8.8	1.5 = 1/10 M.L.D.	Left thyroid gland, diffusely	Repeated in 15 and 30 days	Killed, 40th day
10	8.0	2.5 = 1/10 M.L.D.	Both thyroid glands, diffusely	Repeated in 15 days	Killed, 30th day
11	8.6	2.5 = 1/10 M.L.D.	Both thyroid glands, diffusely	Repeated in 15 and 30 days	Killed, 40th day

testicles, respectively, in 3 dogs. The onset of symptoms was earlier and their severity more marked than after the subcutaneous injections. Death occurred in from 48 to 72 hours. The results are given in Table 1. Colloid present in all the glands; glands in various stages of normal hyperplasia. Active hyperplasia or mitosis not observed. Slight desquamation of the epithelial cells, otherwise no noteworthy changes.

EXPERIMENTS WITH TETANUS TOXIN

The tetanus toxin was kept in dry form, as solutions of tetanus toxin are rapidly destroyed by exposure to light, heat, etc., even when allowed to stand under ordinary conditions.¹⁸

Normal healthy dogs were used in all the experiments. The minimal lethal dose (the amount of tetanus toxin that kills a 300-gm. guinea-pig in 4 days)¹⁹ was found to be 0.00001 gm. This is much larger than that for proportionate weight of guinea-pigs. The latter, when injected subcutaneously in the region of the thyroid gland, was not fatal for 18 or 20 days. The dose was gradually increased to 2.8 times the calculated minimal lethal dose of the proportionate weight of guinea-pig. Such a dose of tetanus toxin was fatal in from 4 to 5 days. This was used as a standard and all calculations for subsequent injections were made on this basis.

That tetanus toxin possesses a strong affinity for the cells of the central nervous system has been shown clearly by Wassermann and Takaki.²⁰ Later, Meyer and Ransom²¹ pointed out that the action and the onset of the symptoms following the injections of tetanus toxin depend on the distance the

¹⁸ Kolle and Wassermann, Handb. d. pathogen. Microorganismen, 1913, 4, p. 983.

¹⁹ Rosenau and Anderson, Public Health Bull. 43, Washington, D. C. Bull. 43, Hyg. Lab., Washington, D. C.

²⁰ Berl. klin. Wehnschr., 1898, 35, p. 5.

²¹ Arch. f. exper. Path. u. Pharmacol., 1903, 49, p. 369.

TABLE 2.—(Continued)
RESULTS OF THYROID GLAND ACTIVITY IN TREATING OF TETANUS TOXIN

Weight of Thyroid Gland		Histologic Description of Thyroid Glands
Right (gm.)	Left (gm.)	
8.0	8.5	Colloid moderate, hyperplastic type. Normal.
3.8	4.2	Colloid type. Normal.
6.8	6.4	Colloid type. Normal.
4.4	4.1	Colloid marked hyperplastic type. Hemorrhages. Desquamation of epithelium. Colloid partly absorbed. Vacuoles.
3.6	3.8	Colloid slight hyperplastic type. Desquamation of epithelium (slight).
5.8	7.2	Colloid type. Left: some hemorrhage. Less colloid?
8.6	8.8	Colloid marked hyperplastic type. Alveoli shrunken. Less colloid. Some desquamation of epithelium.
6.5	9.2	Colloid slight hyperplastic type. Left: hemorrhages. Desquamation of epithelium. Alveoli shrunken, empty. Less colloid.
6.4	8.2	Colloid marked hyperplastic type. Left: thrombus. Hemorrhage. Little colloid. Marked desquamation.
8.0	8.2	Colloid marked hyperplastic type. Alveoli shrunken. Epithelium small. Irregular. Desquamation of epithelium.
9.2	9.5	Colloid moderate hyperplastic type. Hemorrhages. Alveoli shrunken. Amount of colloid decreased. Marked desquamation of epithelium.

toxin has to travel along the motor nerve sheaths to reach the ganglion cells of the central nervous system, and that such results would vary to a certain extent with the location of the injection and the proximity and the amount of nervous tissue present at the site of the injections. In these experiments the amount to be injected was dissolved in 2.5 cc. of physiologic salt solution. The results and data are give in Table 2.

Controls.—Three dogs were weighed and the calculated minimal lethal dose of tetanus toxin was injected into the subcutaneous tissue in the region of the thyroid gland. Twitching and stiffness of the muscles appeared on the 3rd day, followed by typical convulsions within the next 24 hours. Convulsions seldom lasted more than 24 hours before death. The thyroid glands were removed immediately after death and sections prepared for microscopic examination. Glands of the colloid type. The colloid took the stain uniformly. No desquamation or active hyperplasia of the epithelium. No masses of the epithelium.

Minimal Lethal Dose Injected Into Both Thyroid Glands.—Three dogs of approximately uniform size were weighed and prepared for operation. In Dogs 4 and 5 the calculated dose was injected into both thyroid glands as diffusely as possible with the least possible traumatism and the wound closed with the usual aseptic precautions. In Dog 6 the calculated dose was injected into the left thyroid gland. The results are given in Table 2. The symptoms of tetanus appeared from the 4th to the 5th day. This is from 24 to 48 hours later than in the control animals which were injected subcutaneously in the region of the thyroid gland. These symptoms were followed by the usual typical convulsions and death within the next 24 hours; that is, from the 5th to the 6th day. In Dog 6 the injected gland (left) was considerably increased in weight. The classification of the glands as to type is accordance with the microscopic examination and other data is given in Table 2. Microscopic examination: Multiple hemorrhages in the injected thyroid glands.

No necrosis in any of the sections. No change in the amount or in the staining properties of the colloid. Some desquamation of the epithelium from the walls of the alveoli, but no evidence of any active hyperplasia in the injected glands. No mitosis of epithelium.

Repeated Subminimal Lethal Doses.—Repeated subminimal lethal doses of tetanus toxin were injected diffusely into the thyroid glands of 5 dogs. In Dogs 7, 10, and 11, the injections were made into both the right and left thyroid glands. These injections were repeated at intervals of 2 weeks. For these subsequent injections the thyroid glands were always exposed under ether anesthesia through an incision made in the scar tissue of the previous operation. The dogs were killed with ether at intervals ranging from 20 to 40 days after the initial injection. In Dogs 8 and 9 the injections were made only into the left thyroid gland. The data are given in Table 2. The injected thyroid glands were increased in weight. Microscopic examination: Marked decrease in the amount of colloid in the injected (left) glands. Many of the alveoli had no colloid but were partly filled with desquamated epithelial cells. Some hemorrhage and edema in some of the sections. No evidence of any active hyperplasia of the epithelium. No mitosis.

DISCUSSION OF THE RESULTS

It is evident that my results do not confirm the reports made by Remedi. He stated that the injection of diphtheria and tetanus toxin directly into the thyroid gland stimulated the thyroid glands to an increased formation of colloid which neutralized the action and decreased the symptoms produced by these toxins. I found that the minimal lethal dose of diphtheria toxin was invariably fatal in from 1 to 2 days sooner than when injected subcutaneously. This was presumably due to the more rapid entrance of the toxin into the circulation when injected directly into the vascular gland. In the case of tetanus toxin, the dogs that were injected diffusely in the thyroid gland survived those injected subcutaneously in the region of the thyroid gland by 1 or 2 days. There were no evidences of any histologic changes or any activity of the thyroid gland to account for this apparent decrease in toxicity on the part of the tetanus toxin when injected directly into the thyroid gland. Probably the more rapid absorption of the toxin into the blood when injected directly into the thyroid gland would cause more of the tetanus toxin to be bound in such organs as the liver, kidney, and spleen, or there may be fewer routes of travel along motor nerve sheaths from the thyroid gland.

The early increase in weight noticed in the thyroid glands that were injected is explained by the congestion and edema following the injection. There were no changes in the amount of colloid or in the character of the epithelium following single injections. It was only after a few repeated injections that a decrease occurred in the amount of colloid. Six repeated subminimal lethal doses when injected diffusely into the thyroid gland during a period of 3 months caused nearly com-

plete absorption of the colloid. This indicates that the colloid in the alveoli is absorbed under certain conditions, but this absorption does not take place as rapidly as some have contended. According to Bensley,⁹ the colloid in the alveoli represents a reserve supply of the product of the internal secretion of the thyroid gland, and this supply may be slowly absorbed when the normal production by the epithelial cells is decreased by such conditions as cachexia, toxemia, etc. Probably under the conditions established here, when the glands are injected repeatedly with the diphtheria and tetanus toxin, the epithelial cells are exposed to a repeated intoxication. Under such conditions, there would be a decrease in the active production of colloid by the epithelial cells, so that a gradual absorption of the reserve supply of colloid takes place.

Active hyperplastic changes were not found in any of the thyroid glands. This even applies to those glands which were injected as many as 6 times. These results agree with those of Emge.¹¹ There was a marked desquamation of the epithelium, especially noticeable in those thyroid glands which were injected repeatedly with subminimal lethal doses of diphtheria and tetanus toxin. This was presumably due to the continual intoxication and traumatism following these injections.

SUMMARY

The thyroid gland has no detoxicating properties that can be demonstrated by the injection of diphtheria and tetanus toxin directly into the thyroid gland.

A minimal lethal dose of diphtheria toxin, when injected into the thyroid gland, killed from 1 to 2 days sooner than when injected into the subcutaneous tissue.

The symptoms were just as marked in those dogs injected directly in the thyroid gland as in those which were injected subcutaneously.

The injected gland showed an increase in weight in early fatal cases due to congestion and edema.

Microscopic examination did not show any increase in the secretion of colloid as Remedi stated. Repeated injections into the thyroid gland caused an absorption of the colloid from the alveoli.

Active hyperplastic changes of the epithelium as described by Farrant were not evident in these glands. This was true both after single and repeated injections into the thyroid gland.

Repeated subminimal lethal doses of diphtheria and tetanus toxin, when injected diffusely into the thyroid gland, caused a desquamation of the epithelium from the walls of the alveoli.

AN EPIDEMIC OF ACUTE DIARRHEA APPROACHING IN SOME CASES A MILD DYSENTERY *

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The epidemic, which occurred around St. Anne de Bellevue, commenced in the second week of February, 1916, with cases of acute diarrhea. Many of these were not reported, the majority clearing up after the application of simple home remedies. The six cases observed by us readily yielded after purgation with castor oil and the institution of a proper diet. During March the cases reported were decidedly more severe. After the first week in April no new cases developed. A noticeable feature of the outbreak was that the majority of cases occurred among women students, and the worst cases among those whose resistance was low. Clinically, the cases may be outlined in the following subdivisions:

Simple Diarrhea.—These cases, as stated, occurred during February. The symptoms were few. There was usually a sudden onset, accompanied sometimes by vomiting. The stools averaged 6 to 8 a day. They were watery and foul-smelling with little mucus and no blood. No tenesmus and few cramps. Patients somewhat pale; complained of weakness and tiredness, and of impaired appetite. Pulse slightly accelerated, temperature normal throughout, urine and blood normal. These cases, when untreated, showed a tendency to recur at intervals of from 3 to 4 days, the attacks lasting in each case from 2 to 3 days.

Dysenteric Diarrhea.—In some cases this was of sudden onset, in others it was a gradual development from a pre-existing diarrhea. Symptoms were more urgent. About 10 stools a day, at first watery and brown, later becoming green, with considerable mucus and streaks of blood; severe cramps before defecation, little tenesmus. Patients were pale and slightly drawn of face, and complained of headache and drowsiness. Mouth foul and breath offensive; nausea and vomiting; abdomen slightly distended with gas and sensitive to pressure over course of large bowel. Pulse rapid; temperature 100-101. Urine and blood normal. These cases yielded as a rule to diet, bowel lavage, and intestinal antiseptics.

Mild Dysentery.—These cases were not reported at once and consequently were not treated for sometimes as long as 3 weeks. When first seen, the patients presented symptoms of an acute bowel infection,—flushed face, pinched eyes, dry mouth, offensive breath; abdomen distended and sensitive; frequent cramps and some tenesmus; 10 to 12 stools a day, green in color, with mucus and blood; pulse 100; temperature 102-103. The urine in one case showed albumin; the blood in 3 cases showed a polymorphonuclear leukocytosis (count—10,000).

* Received for publication October 18, 1916.

These cases cleared up as a rule in from 8 to 10 days under treatment similar to that for dysenteric diarrhea. One case ran a course similar to paratyphoid. Another developed jaundice 2 weeks after an attack of dysentery. Two cases recurred about 3 weeks after the initial attack and ran a similar, tho milder, course.

TECHNIC

In the cases observed by us (A, B, C, D, E, and F), samples of stools were obtained before any treatment was instituted, sent immediately to the laboratory, and examined almost at once.

Media.—As an alternative to the Conradi-Drigalski medium, which we could not use for lack of crystal violet, 5 drops of a 5% solution of carbolic acid were added to the media employed on pouring the plates. In differentiating the cultures secured, the following media were employed: beef peptone agar and beef peptone gelatin made according to the standard methods;¹ litmus milk, a solution of litmus being sterilized separately and added in sufficient quantity to the milk tubes immediately before inoculation; and beef broth made with Liebig's extract. Dunham's solution for determination of indol-production was prepared as follows:

Witte's peptone	10 gm.	} filtered, tubed, and sterilized.
Sodium chlorid	5 gm.	
Distilled water	1000 c.c.	

After inoculation the tubes were incubated at 37 C. for 6 days and then tested for indol by the Ehrlich method (which gives much more satisfactory results than can be obtained by the use of sulfuric acid and sodium nitrite). Two solutions were used:

- (a) P-dimethyl-amido-benzaldehyde 4 parts
- Absolute alcohol 380 parts
- Concentrated HCl 80 parts
- (b) Saturated aqueous solution of potassium persulfate.

To 10 c.c. of the culture solution 5 c.c. of (a) are added and 5 c.c. of (b). The presence of indol is indicated by the appearance of a cherry-red color almost immediately. McConkey has obtained satisfactory results using 1 c.c. of each of the reagents (a) and (b).

Sugar Reactions.—For these the Smith fermentation tube was used. Peptone salt solution as used for the indol test was prepared and sterilized. The sugars were each made up in a 10% solution with distilled water and after intermittent sterilization, were added to the tubes immediately before inoculation, in such rates as would provide a resulting 1% sugar solution in peptone broth. The inoculations were made from a young peptone-broth culture of the organism.

In 5 of the 6 cases, mucus flakes were present, particularly in Sample D. Pieces of mucus were picked out with a platinum loop, shaken in sterile water, and smeared over the surface of the various media. Traces of the stools were also diluted in broth, plated, and incubated at 37 C., gelatin plates at room temperature. After 24 hours colonies thought worthy of investigation were isolated and streaked on beef peptone agar. Because of the close association of colon organisms with intestinal troubles and on account of the diagnosis of some of the cases as acute diarrhea, we subcultured not only those colonies which resembled the growth of *B. dysenteriae*, but also representatives

¹ Standard Methods of Water Analysis, 1912, pp. 80, 124.

of any other colonies which appeared to be common. Thus the cultural work resolved itself into a general bacteriologic examination of the stools; but the clinical aspects of the cases seemed sufficient warrant for the methods followed.

RESULTS OF CULTURAL STUDIES

A.—The sample from this case yielded pure cultures of *B. coli communior* (Durham).

B.—Each of the cultures here obtained, one excepted, yielded *B. coli communis* (Escherich). The exception, from an 18-hour-old agar culture, under the microscope appeared as a coccus varying to a short thick rod, 0.5 to 1 mm. long, sometimes showing bipolar staining with methylene blue, nonmotile, and gram-positive. Litmus milk,—no coagulation in 6 days, coagulation in 1 month. Beef peptone agar slant,—after 18 hours at 37 C., scanty spreading flat growth; medium unchanged. Litmus agar with mannite, maltose, or saccharose, acid. Glucose, lactose, saccharose, mannite, and maltose broths fermented with production of acid but no gas. Indol not produced.

C.—This case was a severer one. While strains of the colon type were isolated, several colonies which resembled *B. dysenteriae* were transferred to agar slants. Microscopically the organism was a short thick rod resembling the colon bacillus, the majority twice as long as broad, tho longer forms were present, average length 1.5 microns. In a 20-hour-old culture from beef peptone agar at 37 C. no motility was observed, but oscillations were noted resembling the action of the needle of a compass. This bacillus stained with the ordinary anilin dyes and was gram-negative. Agar plates,—20-hour-old colonies from 1 to 2 mm. in diameter, bluish by transmitted light, slightly raised.² Aesculin agar plates,—20-hour-old colonies 1 mm. in diameter, distinctly blue or transparent by transmitted light. Beef peptone agar,—after 24 hours moderate spreading growth, lustrous, flat, bluish by transmitted light; medium unchanged. Gelatin stab,—no liquefaction. Beef broth,—clouding, no pellicle, distinct watered-silk appearance, and, on shaking, a bluish iridescence noted on the side of the tube. Potato,—growth scant, spreading, glistening, the color of wheat straw. Litmus milk,—no coagulation; after 4 weeks reaction distinctly blue. Aesculin agar slant,—moderate growth, no black appearance. Hiss tube medium,—moderate growth, flat, iridescent, bluish by transmitted light. Litmus agar with mannite, maltose, or saccharose, acid. Glucose, mannite, lactose (+ —), maltose, and carbolized broth fermented with production of acid but no gas (saccharose fermented, but alkaline in reaction). Indol-positive.

D.—This case was also a severe one. Sample of stool contained much bloody mucus. Two varieties of organisms were common on all plates, one a type of indol-negative colon bacillus, and the other suggesting *B. dysenteriae*. The latter microscopically resembled the culture described from C. Eighteen hours' growth on beef peptone agar showed short stumpy rods from 1 to 1.5 microns long. In hanging drop the same oscillation was observed, and the same reactions with the various stains. Agar plates,—20-hour-old growth at 37 C. showed colonies 1 mm. in diameter, slightly raised, shiny, bluish by transmitted light. Aesculin agar plate,—20-hour-old growth at 37 C. showed colonies from 0.5 to 1 mm. in diameter, transparent to bluish by transmitted light. Beef peptone agar slant,—growth similar to the culture described from Case C. Litmus agar with mannite acid, with maltose and saccharose alkaline. Glucose, mannite, car-

² Harrison and Vanderleek, *Centralbl. f. Bakteriol.*, I, O., 1909, 51, p. 607.

bolized broth fermented with production of acid but no gas. Lactose, maltose, and saccharose fermented, but alkaline in reaction. Indol-negative.

E.—This was not a severe case, and all the organisms isolated proved to be one variety or another of the colon type.

F.—The sample in this case was green, mucous, and bloody. The plates of the various media showed 2 types of organisms, the one a strong indol-producer, conforming with the general tests for the colon type; the other differing in many respects from the organisms described as resembling *B. dysenteriae*. Its presence in some numbers, however, indicated the probability of its having had some relation to the case. Microscopically it appeared as a coccus from 0.5 to 0.75 mm. in diameter, as a diplococcus, and sometimes as masses resembling staphylococci. In the growth from a 20-hour-old culture on beef peptone agar incubated at 37 C. no motility was observed. It stained deeply with Loeffler's methylene blue, and was gram-positive. Beef peptone agar slant,—after 18 hours, moderate growth, discrete colonies, glistening. Beef broth,—moderate growth and clouding. Potato,—scant, barely perceptible growth. Litmus milk,—no coagulation; final reaction blue. Aesculin agar,—growth, but no black reaction. Litmus agar with mannite, maltose, or saccharose, acid. Glucose, mannite, lactose, maltose, and saccharose fermented with production of acid but no gas. Indol-negative.

SUMMARY

Samples of stools from 6 patients were examined bacteriologically, with results indicating the cases to have been of bacillary origin. Sample A yielded apparently pure cultures of *B. coli-communior* (Durham). Sample B gave 2 types of organisms, *B. coli-communis* (Escherich), and an organism bearing certain resemblances to organisms of the dysentery group. Sample C showed strains of the colon group, and colonies agreeing in all cultural respects with *B. dysenteriae* (Flexner). Sample D gave indol-negative strains of the colon group, and colonies resembling closely *B. dysenteriae* Y of Hiss. Sample E yielded one variety or another of the colon type. Sample F gave 2 types of organisms, a colon type, and an organism that resembled *B. dysenteriae* in some of its cultural and biochemical reactions, but that morphologically was a gram-positive coccus or diplococcus.

The organisms isolated resembling the mannite-fermenting strains of *B. dysenteriae* may be classified according to their reactions on litmus sugar media, as follows:³

Litmus Agar	Shiga	Y (Hiss)	Flexner	Strong	Sample B	Sample C	Sample D	Sample F
Mannite....	—	+	+	+	+	+	+	+
Maltose.....	—	—	+	—	+	+	—	+
Saccharose...	—	—	—	+	+	—	—	+

³ Kolle and Wassermann, Handb. d. pathogen. Mikroorganismen, 1909, p. 391. Flexner, System of Medicine, 1907, 2, p. 477. Besson, Practical Bacteriology, Microbiology, and Serum Therapy, 1913, p. 356. Remlinger and Dumas, Ann. de l'Inst. Pasteur, 1915, 29, p. 498. D'Herelle, Ann. de l'Inst. Pasteur, 1916, 30, p. 145.

The same organisms may be classified according to sugar reactions and indol-production as follows:

Strain	Glucose	Lactose	Saccharose	Maltose	Mannite	Indol
Shiga.....	—	—	—	—
Flexner.....	+	+	+	+
Y (Hiss).....	—	—	+	—
Strong.....	—	+	+	—
Sample B.....	+ A	+ A	+ A	+ A	+ A	—
Sample C.....	+ A	+ Alk	+ Alk	+ A	+ A	+
Sample D.....	+ A	+ Alk	+ Alk	+ Alk	+ A	—
Sample F.....	+ A	+ A	+ A	+ A	+ A	—

A = acid; Alk = alkaline.

Litmus used as indicator.

The occurrence in the same epidemic of both acute diarrhea and dysentery suggests the possibility that both are due to some variation of the same causal organism.

So far as we are aware this is the first time that organisms belonging to the Shiga group have been isolated as far north as Canada.

This and other studies indicate that we have a B.-dysenteriae group analogous to the B.-coli group.

THE PASSIVE TRANSFERENCE OF NONSPECIFIC ANTIBODIES

PLATE 5

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In a previous communication¹ it was demonstrated that the injection of an antigen made from bacteria grown on serum medium could give rise to nonspecific antibodies. Such antigens, altho apparently washed free from the medium, stimulate the production not only of specific antibodies against the bacteria, but also of antibodies against the protein contained in the medium if the protein is heterologous; that is, derived from a species other than that of the animal used in immunization. These antibodies may react nonspecifically against any bacteria grown on such media. This factor may be the cause of the divergence in results in cross complement-fixation between the meningococcus and the gonococcus, or in that between dissimilar groups of streptococci, or between different organisms when the immune serum used in the tests has been prepared by injecting serum-grown bacteria into rabbits.

The object of this paper is to show that a similar phenomenon occurs in passive sensitization. Immune serum developed against repeated injections of bacteria grown on serum media, when inoculated into guinea-pigs, sensitizes the cells of this animal, not only to the bacteria, but also to the protein present in the medium on which the antigen has been grown.

Our attention was drawn to this fact while we were working with sensitized guinea-pig uteri by the Dale method.² We employed the technic as utilized by R. Weil in his study of pneumococcal sensitization.³ In general the principles of this method are as follows:

A guinea-pig is sensitized by the subcutaneous injection of from 2 to 3 c.c. of the serum to be studied. After from 2 to 6 days the animal is killed and its uterus is removed; either horn is suspended in from 75 to 125 c.c. of Locke's solution. This solution is kept at a constant temperature of from 37 to 40 C. When the antigen is added to the fluid surrounding the uterus, there occurs a greater or lesser degree of contraction, the intensity of which is recorded on a drum covered with smoked paper. As a control, to test the

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¹ Olitsky and Bernstein, *Jour. Infect. Dis.*, 1916, 19, p. 253.

² *Jour. Pharm. and Exper. Therap.*, 1913, 4, p. 167.

³ Weil and Torrey, *Jour. Exper. Med.*, 1916, 23, p. 1.

contractility of the uterine muscle, 0.0002 gm. of ergamine is added; this should give a contraction in a responsive or "live" uterus. After each addition of material to the Locke solution, the fluid is removed, the uterus washed, and fresh solution is added—all these procedures being conducted at a constant temperature.

In our earlier work, while experimenting with the passive transference of antibodies, we noted the following: A rabbit was immunized against bacteria grown on human-serum media. The immunization was effected by repeated injections of the antigen over a long period of time. Blood from this rabbit was injected into a guinea-pig, and after a few days the uterus was removed, suspended in the Dale apparatus, and tested. It was found then that not only did the uterus contract on the addition of antigen (see Fig. 1), but also on the addition of plain human serum (see Fig. 2). Thus we see that the antigen used in immunizing the rabbit had produced serum antibodies as well as specific bacterial antibodies.

A series of experiments was then undertaken with the object of investigating these phenomena.

A strain of *B. typhosus* was employed as antigen because the history of this culture was known: for years it has been transplanted and growing on plain agar. The organism in one instance was allowed to grow on plain agar; in the second, on human-serum agar.

Rabbit 1 received 7 intravenous injections over a period of 12 days, of saline suspensions of typhoid bacilli grown on plain agar. Between the 1st and 2nd injections there was an interval of 7 days, thereafter an injection was given daily. The 1st inoculation was made of dead typhoid bacilli (the growth on 1 agar slant); the subsequent inoculations, of live organisms, starting with 1 and ending with 6 loopfuls. Ten days after the last injection, the animal was bled and the titration of humoral antibodies showed: complement fixation, ++++ in 0.001 c.c. of serum; macroscopic agglutination, positive in 1:400 dilution. Altho the antibody content was low, it was sufficient for our purpose, since the Dale method detects the faintest possible trace of absorbed antibody. This, then, was the source of the "plain-agar typhoid immune serum."

Rabbit 2 was immunized in the very same manner, except that the antigen, altho consisting of the same strain of the typhoid bacillus, was made of the organisms grown on serum media. The first injection consisted of the same strain used in the case of Rabbit 1 grown for 3 generations on human-serum media. The 2nd to the 7th injections consisted of organisms of the 10th to the 15th generation, respectively. Care was always taken to exclude particles of the media from the suspension, which was washed in 0.9% salt solution 4 times. The supernatant fluid after each washing was tested for the presence of serum albumin and the suspension was used only when such test was negative. Ten days after the last injection the animal showed: complement fixation, +++ in 0.001 c.c. of its serum; macroscopic agglutination, positive in a dilution of 1:800. This was the source of the "serum-agar typhoid immune serum."

The experiments from this point were based on the principle that the

cells of the guinea-pig will absorb the antibodies from an immune serum and respond to the addition of a corresponding antigen. Accordingly, the "plain" and the "serum" typhoid immune sera (2.5 c.c.) were injected subcutaneously into guinea-pigs; 5 days later the animals were killed, and the uteri removed and suspended in Locke's solution, and tested.

In the case of the guinea-pig injected with the "plain" typhoid immune serum, it was determined that the uteri had absorbed typhoid antibodies (see Fig. 3a); there was no reaction to serum (see Fig. 3b and c).

In the case of the guinea-pig injected with the "serum" typhoid immune serum, it was determined that the uteri had absorbed typhoid antibodies (see Fig. 4) and at the same time antibodies against human serum (see Fig. 5).

DISCUSSION

It is possible that traces of the media may be carried over in the preparation of the antigens. We have kept this factor in mind and tried as much as possible to eliminate it. The possibility becomes still more remote when the degree of the nonspecific serum reaction is taken into account. It is difficult to conceive that minute traces of the media should so highly immunize the animal as to give the violent reactions noted in our experiments — reactions much more marked than those against the original antigens.

A great deal of evidence is rapidly accumulating showing the effect of media on the biologic variations of bacteria. Differences and changes in carbohydrate fermentation, in virulence, and in growth have been noted — some of these variations becoming permanent. The question arises whether one biotype transferred to a new environment will change its characteristics and become a new biotype developing along a pure line permanently. In our case, the typhoid bacilli growing on serum media absorb certain of the serum elements and become, so to speak, "serum" typhoid organisms. This may give rise to the non-specific factors, for, theoretically, different bacteria may absorb the same chemical molecule from the serum of the same species. Wells and Osborne have recently demonstrated that anaphylactic specificity is dependent on the chemical constitution of the vegetable protein rather than on the species.

Several investigators have reported cross-fixations with different bacteria. In such instances the cross-fixation may be an evidence, not of the identity of the bacterial proteins, but of the identity of the proteins in the media. Whether the protein of the media is carried over into the antigen, or whether the bacteria absorb from the media certain common chemical molecules, we can not say.

The practical point of these experiments is that great care should be exercised in classifying bacteria by the ordinary method of testing against rabbit immune serum.

EXPLANATION OF PLATE

FIG. 1. This guinea-pig was sensitized to rabbit immune serum prepared against bacteria grown on serum media. The uterus shows a contraction on addition of the same antigen.

FIG. 2. The same uterus as in Fig. 1, showing a contraction even more marked on addition of human serum alone. The upper tracing shows the desensitization caused by the initial addition of the antigen, and the control contraction in response to ergamine.

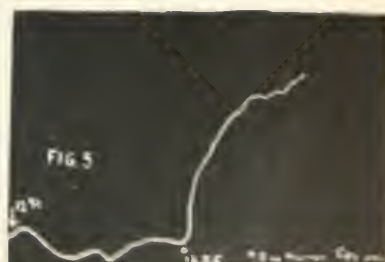
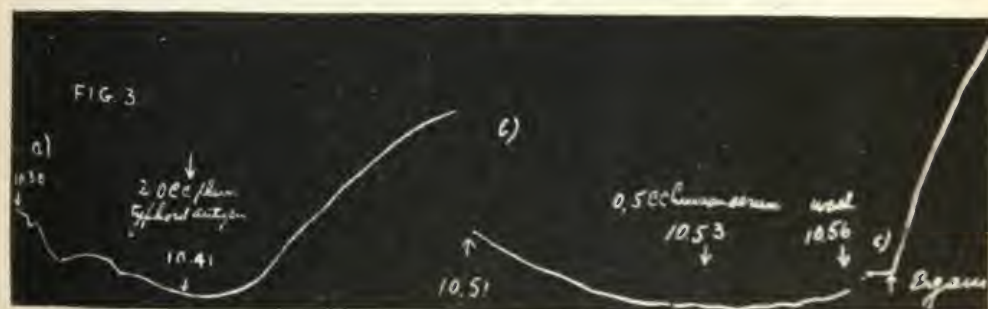
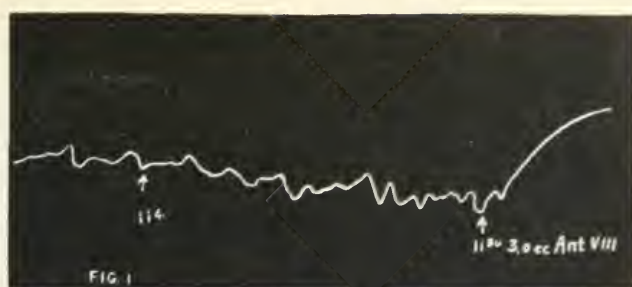
FIG. 3. Uterus of a guinea-pig sensitized to rabbit immune serum prepared against "plain-agar" typhoid antigen (bacteria grown on plain agar).

- a. Contraction in response to similar antigen.
- b. No contraction in response to human serum.
- c. Contraction in response to ergamine, showing uterus to be "alive."

FIG. 4. Guinea-pig uterus sensitized to rabbit immune serum developed against serum-grown typhoid antigen. Shows contraction in response to the same antigen.

FIG. 5. Same uterus as in Fig. 4, showing contraction in response to human serum.

PLATE 5



COMPARATIVE STUDY OF BACILLUS ANTHRACIS-SYMPOMATICI AND ALLIED ORGANISMS WITH RESPECT TO GAS- PRODUCTION *

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The question of the differentiation of the bacillus of symptomatic anthrax from that of malignant edema often arises. The fact that both possess the ability to produce gas when grown in carbohydrate-containing media, suggested that this characteristic might be used in differentiation. A study of the literature revealed such a paucity of data in this field that further work seemed necessary.

In this communication attention has been confined largely to the production of carbon dioxid and hydrogen and to their ratios. Work has also been done on the production of hydrogen sulfid and on several other biochemical characteristics, such as the production of methyl mercaptan, indol, and acids, and the action on nitrates. This work will be the substance of a later report.

The sources of the various strains are recorded in Table 1. Attention was paid especially to the two organisms mentioned, but several allied organisms were included for the sake of comparison. A very active gas-producing strain of *B. coli* was used as a control.

Sugar-free broth was prepared by allowing an active strain of *B. coli* to grow in regular beef infusion for 18 hours. The medium was then boiled 20 minutes and filtered, and 2% Witte's peptone and 0.5% sodium chlorid added. It was then titrated and adjusted to a reaction neutral to phenolphthalein.¹ The medium was sterilized fractionally for 30 minutes at 100 C. on each of 3 successive days.

Control inoculations in this sugar-free broth were made with a strain of *B. coli* which produced large quantities of gas in media containing dextrose, levulose, galactose, arabinose, mannose, rhamnose, xylose, lactose, maltose, saccharose, raffinose, sorbite, dulcite, mannite, and salicin; and also with a strain of *B. mucosus-capsulatus* that produced gas in media containing dextrose, levulose, galactose, mannose, lactose, maltose, saccharose, dextrin, and glycerin. In these controls no gas was produced during 10 days' incubation.

This broth was then used as the bearer of the various carbohydrates. These were added in 1% proportion and the resulting media, in 250-c.c. flasks, were heated 30 minutes in flowing steam, filled into sterile fermentation tubes, heated

* Received for publication October 18, 1916.

¹ Standard Methods of Water Analysis, 1912.

TABLE 1
THE SOURCES OF THE STRAINS STUDIED

Organism and Strain No.		Source
B. anthracis-symptomatici	2	Blackleg vaccine from U. S. Dept. of Agriculture, Washington, D. C.
	3	An American commercial blackleg vaccine virus
	4	A tube of blackleg virus (desiccated blackleg meat) received from Dr. G. W. Dunphey, state veterinarian, Lansing, Mich.
	5	Culture from symptomatic anthrax, received from Dr. V. A. Moore, Cornell University, Ithaca, N. Y.
	6	A French commercial blackleg vaccine virus
	10	A French commercial blackleg vaccine virus
	12	An American commercial blackleg vaccine virus
B. oedematis-maligni	48	Culture received from an American commercial laboratory
	60	Culture received from Dr. F. G. Novy, University of Michigan, Ann Arbor
	421	Culture received from the culture bureau of the American Museum of Natural History, New York City
B. oedematis-maligni II	485	Culture received from the culture bureau of the American Museum of Natural History, New York City
	558	Culture received from the culture bureau of the American Museum of Natural History, New York City
	560	Culture received from Dr. F. G. Novy
B. botulinus	1	Culture received from Dr. Novy
Ghon-Sachs bacillus	1	Culture received from Dr. Novy
B. coli (De Snoo)	1	Culture received from Dr. K. F. Meyer, University of California, Berkeley
		Culture received from Dr. Poels, Holland state serum laboratory, Rotterdam

in the tubes for another 30 minutes in flowing steam, and then incubated 4 days to reveal contamination. It was found by this method that sterilization was accomplished with slight hydrolysis of the various carbohydrates.

Baseless fermentation tubes, holding 12 c.c. of media when filled to a convenient height, were used. These were readily handled in special racks holding 18 tubes each. They stood in such position that the closed arm was always upright and could not be jarred loose from this position during handling of the racks.

The following carbohydrates, alcohols, and glucosids² were used:

Monosaccharids—dextrose, levulose, galactose, arabinose, mannose, rhamnose, xylose.

Disaccharids—lactose, maltose, saccharose.

Trisaccharid—raffinose.

Polysaccharids—dextrin, inulin, glycogen, starch.

Alcohols—the trihydric, glycerin; the pentahydric, adonite; the hexahydric, sorbite, dulcitol, and mannitol.

Glucosids—salicin, amygdalin, phlorizin.

The polysaccharids dissolved with some difficulty. Soluble starch, according to Lintner, was employed. The carbohydrates were of the best quality obtainable.

The experiment was made completely twice over, as indicated in Tables 2 and 3. In the first trial, as seed of the various strains, 4-day-old cultures in Martin's broth³ were used. These strains had been carried as stock cultures in Martin's broth under hydrogen, the interval of transplant having been

²Hawk, Physiological Chemistry, 1914, p. 25. Eyre, Technique of Bacteriology, 1913, p. 178.

³ Besson, Bacteriology, Microbiology and Serum Therapy, 1913, p. 33.

6 days. In the second trial, the seed cultures had been trained for 2 generations in the plain sugar-free broth prepared. Otherwise, the conditions were maintained as nearly as possible the same in both experiments.

Inoculation of fermentation tubes was made by the pipet method, 4 drops of the sediment emulsion being used, which, in the case of the spore-bearers, contained enormous numbers of spores.

Readings were taken every 24 hours, and gas levels marked with water-proof hard-drying carbon ink. Readings were made for at least 11 consecutive days. Gas in the closed arm is indicated in the tables in percentages of total height of column. Less than 5% is designated + because amounts less than that can be estimated only approximately. Readings were made by means of a finely graduated gasometer at the back of the tube and to the bottom of the meniscus. Carbon dioxid was determined by absorption with 2% sodium-hydroxid solution.

In figuring out the so-called gas coefficients, the formula CO_2/H was used. This directs special attention to the carbon dioxid produced, since the resulting figure gives at a glance a comparative estimate of the carbon dioxid in proportion to the residual gas, which is largely hydrogen, tho there is probably some methane in it also.⁴ The greater the factor, the greater the proportionate amount of carbon dioxid produced.

For all practical purposes the coefficients obtained with gas of less than 10% mean little, unless there is a decided reduction in volume when the carbon dioxid is absorbed. Even a slight reduction—1%—in the amount of gas in the closed arm is detectable and measurable, but most emphasis should be placed on the coefficients obtained from gas produced in larger amounts.

During the sodium-hydroxid treatment, reduction of the gas volume present, in the small as well as in the larger amounts, indicates the presence of carbon dioxid and is of value as suggesting the splitting up of carbohydrate material. In this experiment in which so-called sugar-free broth was used as control on the sugar media, gas was produced by the majority of the strains. In no case was there even a suggestion of reduction of volume during the sodium-hydroxid treatment. There was no carbon dioxid present, but the gas formed was explosive when mixed with air. In the first trial, conducted like the second except for the difference in seed cultures, the same strain of *B. coli* and several other strains produced no gas from the broth during 11 days' incubation. In the second trial, for which the seed used had been grown and trained (with some difficulty) in the sugar-free broth, colon bacilli, as well as the majority of the spore-bearing anaerobes, produced gas from

⁴ Besson, *Bacteriology, Microbiology and Serum Therapy*, 1913, p. 555.

TABLE 2
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY BACILLUS ANTHRACIS-
SYMPTOMATICI AND ALLIED ORGANISMS *

Strain	Dex- trose	Levu- lose	Galac- tose	Arabi- nose	Man- nose	Xylose	Lac- tose	Mal- tose	Saccha- rose	Raffi- nose	Dex- trin	Inulin
<i>Bacillus anthracis-symptomatici</i>												
2	0 10 + 10 7 + 10 18 + 10 20 + 10 20	+ 22 22 + 22 25 + 22 25 + 22 25 + 22 25	+ 10 10 + 10 10 + 10 22 + 10 22 + 10 22	0 8 + 8 8 + 8 8 + 8 8 + 8 8	+ 10 10 + 10 10 + 10 10 + 10 10 + 10 10	+ 5 5 + 5 5 + 5 5 + 5 5 + 5 5	+ 12 12 + 12 12 + 12 12 + 12 12 + 12 12	0 20 5 20 10 20 12 20 15 20	0 10 5 10 7 10 10 10 10 10	0 5 + 5 5 + 5 5 + 5 5 + 5 5	0 10 + 10 10 + 10 10 + 10 10 + 10 10	25 30 27 30 30 30 30 30 30 30
	$x = \frac{10}{10} = 1$	$x = \frac{17}{8} = 2.12$	$x = \frac{5}{55} = 0.09$	$x = \frac{3}{5} = 0.6$	$x = \frac{8}{27} = 0.29$	$x = \frac{2}{3} = 0.67$	$x = \frac{0}{12} = 0$	$x = \frac{20}{8} = 2.5$	$x = \frac{3}{7} = 0.4$	$x = \frac{2}{5} = 0.4$	$x = \frac{0}{10} = 0$	$x = \frac{5}{25} = 0.2$
3	+ 15 15 + 18 15 + 18 20 + 20 23 + 20 25	0 30 10 30 15 30 18 30 25 30	0 5 + 5 5 + 5 5 + 5 5 + 5 5	0 10 + 10 10 + 10 10 + 10 10 + 10 10	0 7 + 7 7 + 7 7 + 7 7 + 7 7	+ 5 5 + 5 5 + 5 5 + 5 5 + 5 5	0 7 + 7 7 + 7 7 + 7 7 + 7 7	0 43 7 50 15 55 20 45 25 62	0 8 + 8 8 + 8 8 + 8 8 + 8 8	0 5 + 5 5 + 5 5 + 5 5 + 5 5	0 + 0 + + + + + + +	0 0 0 0 0 0 0 0 0 0
	$x = \frac{18}{5} = 3.6$	$x = \frac{20}{10} = 2$	$x = \frac{8}{52} = 0.15$	$x = \frac{5}{5} = 1$	$x = \frac{2}{5} = 0.4$	$x = \frac{1}{4} = 0.25$	$x = \frac{2}{5} = 0.4$	$x = \frac{50}{12} = 4.17$	$x = \frac{3}{5} = 0.6$	$x = \frac{2}{8} = 0.25$	$x = \frac{0}{+} = 0$	$x = 0$
4	0 7 + 12 12 + 12 12 + 15 15 + 22 22	+ 8 8 + 8 8 + 8 8 + 8 8 + 8 8	+ 7 7 + 7 7 + 7 7 + 7 7 + 7 7	0 7 + 7 7 + 7 7 + 7 7 + 7 7	0 8 + 8 8 + 8 8 + 8 8 + 8 8	+ 10 10 + 10 10 + 10 10 + 10 10 + 10 10	0 7 + 7 7 + 7 7 + 7 7 + 7 7	10 45 10 48 17 48 30 45 35 52	0 17 + 17 17 + 17 17 + 17 17 + 17 17	+ 12 12 + 12 12 + 12 12 + 12 12 + 12 12	0 8 + 8 8 + 8 8 + 8 8 + 8 8	10 10 10 10 10 10 10 10 10 10
	$x = \frac{15}{7} = 2.14$	$x = \frac{4}{4} = 1.0$	$x = \frac{7}{20} = 0.35$	$x = \frac{3}{7} = 0.43$	$x = \frac{3}{5} = 0.6$	$x = \frac{3}{7} = 0.43$	$x = \frac{2}{5} = 0.4$	$x = \frac{40}{12} = 3.5$	$x = \frac{4}{13} = 0.3$	$x = \frac{2}{10} = 0.2$	$x = \frac{3}{5} = 0.6$	$x = \frac{2}{8} = 0.25$
5	0 40 + 42 45 + 45 45 + 45 45 + 45 45	0 42 15 42 20 45 30 45 40 45	+ 8 8 + 8 8 + 8 8 + 8 8 + 8 8	0 15 10 15 12 15 15 15 15 15	0 7 + 7 7 + 7 7 + 7 7 + 7 7	+ 8 8 + 8 8 + 8 8 + 8 8 + 8 8	0 10 + 10 10 + 10 10 + 10 10 + 10 10	0 48 20 50 30 50 35 50 40 50	0 25 5 25 7 25 10 25 10 25	0 8 + 8 8 + 8 8 + 8 8 + 8 8	0 5 + 5 5 + 5 5 + 5 5 + 5 5	+ 10 10 + 10 10 + 10 10 + 10 10 + 10 10
	$x = \frac{28}{17} = 1.65$	$x = \frac{35}{10} = 3.5$	$x = \frac{4}{6} = 0.67$	$x = \frac{5}{10} = 0.5$	$x = \frac{2}{5} = 0.4$	$x = \frac{0}{8} = 0$	$x = \frac{2}{8} = 0.25$	$x = \frac{37}{13} = 2.85$	$x = \frac{5}{20} = 0.25$	$x = \frac{0}{8} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{2}{8} = 0.25$
6	0 70 18 75 30 75 45 80 55 80 65 80	0 22 5 22 10 27 12 27 15 27 20 27	+ 12 12 10 12 10 12 10 13 10 13 10 13	0 5 + 5 5 + 5 5 + 7 7 + 7 7 + 7 7	+ 20 20 15 20 17 20 18 20 18 20 20 20	0 8 + 8 8 + 8 8 + 8 8 + 8 8 + 8 8	0 8 + 8 8 + 8 8 + 8 8 + 8 8 + 8 8	0 25 5 25 8 28 10 25 15 35 20 35	0 7 + 7 7 + 7 7 + 7 7 + 7 7 + 7 7	0 5 + 5 5 + 5 5 + 5 5 + 5 5 + 5 5	0 8 + 8 8 + 8 8 + 8 8 + 8 8 + 8 8	0 7 + 7 7 + 7 7 + 7 7 + 7 7 + 7 7
	$x = \frac{60}{20} = 3$	$x = \frac{19}{8} = 2.37$	$x = \frac{6}{7} = 0.84$	$x = \frac{2}{5} = 0.4$	$x = \frac{7}{13} = 0.53$	$x = \frac{2}{6} = 0.33$	$x = \frac{1}{7} = 0.14$	$x = \frac{25}{10} = 2.5$	$x = \frac{2}{5} = 0.4$	$x = \frac{0}{5} = 0$	$x = \frac{3}{5} = 0.6$	$x = \frac{5}{15} = 0.33$
10	0 15 + 18 18 + 18 18 + 100 100 + 100 100	0 50 5 50 12 50 45 50 45 50	0 + + + + + + + + +	0 7 + 7 7 + 7 7 + 7 7 + 7 7	+ 10 10 + 10 10 + 10 10 + 10 10 + 10 10	0 7 + 7 7 + 7 7 + 7 7 + 7 7	0 10 + 10 10 + 10 10 + 10 10 + 10 10	0 20 5 20 8 25 10 20 15 20	0 10 + 10 10 + 10 10 + 10 10 + 10 10	0 5 + 5 5 + 5 5 + 5 5 + 5 5	0 35 35 35 35 35 35 35 35 35	+ 7 7 + 7 7 + 7 7 + 7 7 + 7 7
	$x = \frac{20}{80} = 0.25$	$x = \frac{23}{27} = 0.85$	$x = 0$	$x = \frac{2}{5} = 0.4$	$x = \frac{2}{8} = 0.25$	$x = \frac{2}{5} = 0.4$	$x = \frac{3}{7} = 0.43$	$x = \frac{12}{8} = 1.5$	$x = \frac{0}{10} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{2}{33} = 0.06$	$x = \frac{3}{60} = 0.05$
12	0 5 + 5 5 + 5 5 + 95 95 + 95 95	0 24 10 24 15 25 20 33 20 33	0 8 + 8 8 + 8 8 + 8 8 + 8 8	0 10 + 10 10 + 10 10 + 10 10 + 10 10	0 7 + 7 7 + 7 7 + 7 7 + 7 7	+ 7 7 + 7 7 + 7 7 + 7 7 + 7 7	0 30 + 30 30 + 30 30 + 30 30 + 30 30	0 25 7 25 10 25 15 25 20 25	+ 18 18 5 18 5 18 5 18 7 18	0 5 + 5 5 + 5 5 + 5 5 + 5 5	0 0 0 0 0 0 0 0 0 0	0 15 10 15 12 15 12 15 12 15
	$x = \frac{10}{85} = 0.12$	$x = \frac{18}{15} = 1.2$	$x = \frac{3}{5} = 0.6$	$x = \frac{3}{7} = 0.43$	$x = \frac{2}{5} = 0.4$	$x = \frac{1}{6} = 0.14$	$x = \frac{5}{25} = 0.2$	$x = \frac{15}{10} = 1.5$	$x = \frac{3}{15} = 0.2$	$x = \frac{0}{5} = 0$	$x = 0$	$x = \frac{15}{50} = 0.3$

* Readings made every 24 hours for 11 days. Amount of gas produced indicated in percentages. Less than 5% is designated +.

Uninoculated control tubes of all media were negative throughout the 11 days' incubation.

TABLE 2.—Continued

COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS-SYMPOMATICI* AND ALLIED ORGANISMS *

Glyco- gen	Starch	Glyc- erin	Ado- nite	Sor- bite	Dul- cite	Man- nite	Rham- nose	Salic- in	Amyg- dalin	Phlori- zin	Carbohy- drate-free Broth
<i>Bacillus anthracis-sympomatici</i>											
0 + + + 5	0 0 5 5 5	0 + + 5 5	0 + + 5 5	0 7 10 10 13 15	0 7 5 5 5	0 7 5 5 7	0 7 10 10 10 10	+ 15 17 20 22 25	+ 7 5 5 7 7	0 0 0 0 0 0	0 0 0 0 0 0
$x = \frac{0}{5} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{2}{40} = 0.05$	$x = \frac{1}{6} = 0.16$	$x = \frac{10}{13} = 0.77$	$x = \frac{2}{5} = 0.4$	$x = \frac{1}{6} = 0.16$	$x = \frac{1}{9} = 0.11$	$x = \frac{7}{20} = 0.34$	$x = \frac{2}{5} = 0.4$	$x = 0$	$x = 0$
0 + + + 5	0 + 5 5 5	0 10 12 12 12	0 + 5 5 5	0 8 12 15 17 18	0 7 5 5 5	0 7 5 5 5	0 8 5 7 8 8	+ 20 25 30 30 32	+ 7 5 7 7 7	0 0 0 0 0 0	0 0 0 0 0 0
$x = \frac{0}{5} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{3}{12} = 0.25$	$x = \frac{1}{6} = 0.16$	$x = \frac{13}{15} = 0.87$	$x = \frac{1}{6} = 0.16$	$x = \frac{0}{8} = 0$	$x = \frac{0}{7} = 0$	$x = \frac{11}{24} = 0.46$	$x = \frac{0}{10} = 0$	$x = 0$	$x = 0$
0 + + + 5	0 5 7 10 10	0 12 7 8 10	0 12 5 7 7	7 25 10 15 18 22	0 7 5 7 7	0 7 5 7 7	0 7 5 10 7	+ 15 20 25 25 30	0 5 5 5 5	0 0 0 0 0	0 0 0 0 0
$x = \frac{0}{5} = 0$	$x = \frac{2}{8} = 0.25$	$x = \frac{2}{10} = 0.2$	$x = \frac{1}{6} = 0.16$	$x = \frac{13}{14} = 0.9$	$x = \frac{0}{7} = 0$	$x = \frac{1}{9} = 0.11$	$x = \frac{0}{5} = 0$	$x = \frac{8}{24} = 0.33$	$x = \frac{0}{5} = 0$	$x = 0$	$x = 0$
+ + + 5 5	+ 5 7 8 8	0 8 8 8 8	+ 5 5 5 5	0 15 5 20 20 14	0 7 + 6 6 6	0 7 + 6 6 6	0 11 0 8 10 10	+ 35 20 25 28 30 35	+ 8 7 7 8 7 7	0 0 0 0 0 0	0 0 0 0 0 0
$x = \frac{0}{5} = 0$	$x = \frac{1}{9} = 0.11$	$x = \frac{0}{8} = 0$	$x = \frac{1}{7} = 0.14$	$x = \frac{10}{10} = 1$	$x = \frac{0}{7} = 0$	$x = \frac{1}{10} = 0.1$	$x = \frac{0}{5} = 0$	$x = \frac{13}{25} = 0.52$	$x = \frac{1}{7} = 0.14$	$x = 0$	$x = 0$
+ + + 5 5	0 8 9 9 8	0 10 + 5 5	0 10 + 8 10	+ 48 12 13 18 22 30	0 7 + 6 6 6	0 7 + 6 6 6	+ 7 7 7 7 7	0 5 + 5 5	0 7 + 5 7 7	0 0 0 0 0 0	0 + + + +
$x = \frac{0}{5} = 0$	$x = \frac{1}{8} = 0.13$	$x = \frac{2}{8} = 0.25$	$x = \frac{0}{10} = 0$	$x = \frac{55}{15} = 3.67$	$x = \frac{0}{7} = 0$	$x = \frac{1}{6} = 0.6$	$x = \frac{0}{7} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{0}{7} = 0$	$x = 0$	$x = 0$
0 + + + 5	0 7 + 8 8	0 17 10 15 15	+ 7 5 5 7	0 15 5 7 10 11 12	0 7 + 5 7 7	0 7 + 5 7 7	0 9 0 5 7 9 9	0 10 8 10 10 10 20	0 7 + 5 5 7	0 0 0 0 0 0	0 0 0 0 0 +
$x = \frac{0}{5} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{5}{12} = 0.42$	$x = \frac{0}{7} = 0$	$x = \frac{8}{10} = 0.8$	$x = \frac{0}{7} = 0$	$x = \frac{0}{9} = 0$	$x = \frac{0}{10} = 0$	$x = \frac{2}{20} = 0.1$	$x = \frac{0}{7} = 0$	$x = 0$	$x = 0$
0 + + + 5	0 7 + 8 8	0 8 + 8 8	0 8 + 8 8	+ 10 5 5 8 10	0 7 + 5 5 5	0 7 + 5 5 5	0 8 + 5 5 5	0 20 17 20 20 20	0 10 + 10 10 10	0 0 0 0 0 0	0 0 0 0 0 0
$x = \frac{0}{5} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{2}{6} = 0.33$	$x = \frac{2}{6} = 0.33$	$x = \frac{3}{9} = 0.25$	$x = \frac{1}{6} = 0.16$	$x = \frac{0}{7} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{0}{20} = 0$	$x = \frac{0}{10} = 0$	$x = 0$	$x = 0$

TABLE 2.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS-SYMPOMATICI* AND ALLIED ORGANISMS *

Strain	Dex-trose	Levu-lose	Galac-tose	Arabi-nose	Man-nose	Xylose	Lac-tose	Mal-tose	Saccha-rose	Raffi-nose	Dex-trin	Inulin
* <i>Bacillus anthracis-sympomatici</i>												
48	0 + + 5 + 5 + 7 + 7 +	+ 35 13 35 15 40 20 55 27 55 30	0 5 + 5 + 5 + 13 5 13 5	0 8 + 8 + 8 5 8 5 8 5	0 12 + 12 10 12 12 12 12 12 12	+ 13 + 13 5 13 5 13 5 13 5	0 15 + 15 5 15 5 15 5 15 5	0 22 + 25 7 28 10 22 15 22 20	+ 15 13 15 15 15 15 13 15 13 15	+ 5 + 5 5 5 5 5 5 5 5	+ 5 + 5 + 5 + 5 + 5 +	0 8 + 8 5 8 5 8 5 8 5
	$x = -0 = 7$	$x = -0.83 = 30$	$x = -0.3 = 10$	$x = -0.6 = 5$	$x = -0.2 = 10$	$x = -1.6 = 5$	$x = -0 = 15$	$x = -3.4 = 5$	$x = -0.3 = 10$	$x = -0 = 5$	$x = -0 = 5$	$x = -0 = 8$
60	+ 47 12 47 23 50 30 50 38 50 42	5 52 12 52 20 55 30 55 38 55 42	5 10 5 10 8 10 8 10 10 10 10	+ 8 + 10 6 10 7 10 7 10 8	+ 7 + 7 5 7 7 7 7 7 7	+ 7 + 7 + 7 + 7 + 7 5	+ 5 + 5 5 5 5 5 5 5 5	+ 32 53 67 Broken 8 8 8	+ 8 + 8 7 8 8 8 8 8 8	+ 5 + 5 + 5 + 5 + 5 +	0 + + + + + + 7 + 7 +	+ 8 + 8 7 8 8 8 8 8 8
	$x = -6.14 = 7$	$x = -4.5 = 10$	$x = -0.25 = 8$	$x = -0.25 = 8$	$x = -0.16 = 6$	$x = -0.16 = 6$	$x = -0 = 5$		$x = -0.14 = 7$	$x = -0 = 5$	$x = -0 = 7$	$x = -0 = 8$
<i>Bacillus oedematis-maligni</i> , I												
421	+ 25 10 25 15 25 20 25 20 25 20	+ 28 12 28 20 28 25 28 25 28 25	0 5 + 5 + 5 + 7 + 7 5	0 10 0 10 + 10 5 58 7 58 10	0 15 + 15 10 15 12 15 12 15 15	0 7 + 7 + 7 5 7 7 7 7	0 15 + 15 7 15 10 15 10 15 10	0 40 17 42 25 45 30 45 35 45 40	0 7 + 7 + 10 + 12 + 20 5	0 8 + 8 + 8 5 8 5 8 5	0 5 + 5 + 5 + 5 + 5 +	+ 10 + 10 5 10 5 10 5 10 8
	$x = -2.12 = 8$	$x = -1.8 = 10$	$x = -0.4 = 5$	$x = -0.05 = 55$	$x = -0.25 = 12$	$x = -0 = 7$	$x = -0.25 = 12$	$x = -2 = 15$	$x = -0.33 = 15$	$x = -0 = 8$	$x = -0.25 = 4$	$x = -1 = 5$
485	0 15 5 15 8 15 12 18 12 18 15	+ 45 17 45 30 50 35 50 40 50 45	0 7 + 7 5 7 5 7 5 7 5	0 5 0 5 + 5 5 8 5 8 5	0 12 5 12 10 12 10 12 10 12 10	+ 9 + 9 5 9 7 9 9 9 9	0 10 + 10 5 10 5 10 5 10 5	0 28 10 30 15 30 20 30 22 30 25	0 10 0 10 + 10 + 10 + 10 5	0 7 + 7 7 7 7 7 7 7 7	0 28 + 28 + 28 + 28 + 28 25	0 + + + + + + + + + +
	$x = -2 = 6$	$x = -4 = 10$	$x = -0.4 = 5$	$x = -0 = 8$	$x = -0.5 = 8$	$x = -0 = 9$	$x = -0 = 10$	$x = -2 = 10$	$x = -0 = 10$	$x = -0 = 7$	$x = -0.17 = 24$	$x = -0 = +$
558	+ 14 + 15 5 17 10 20 10 20 10	0 17 + 17 5 18 10 22 12 22 15	0 + 0 + + + + + + 4 +	0 10 5 10 5 10 5 10 7 10 7	0 10 5 10 5 10 10 10 10 10 10	+ 5 + 5 + 5 5 5 5 5 5	+ 5 + 7 + 10 5 13 5 13 5	+ 30 10 30 20 30 22 30 25 30 28	0 7 + 7 + 8 + 10 + 10 5	0 5 + 5 5 5 5 5 5 5 5	0 7 0 7 + 7 5 7 5 7 5	0 + 0 + + + + + + + +
	$x = -1 = 10$	$x = -3.4 = 5$	$x = -1 = 2$	$x = -0.25 = 8$	$x = -0.25 = 8$	$x = -0.25 = 4$	$x = -0 = 13$	$x = -2 = 10$	$x = -0 = 10$	$x = -0 = 5$	$x = -0.4 = 5$	$x = -0 = +$
500	+ 8 5 8 5 8 7 8 8 8 8	0 24 5 28 8 30 12 33 15 33 20	5 17 12 17 15 17 15 17 16 17 16	0 + + + + + + + + + +	+ 20 10 20 13 20 16 20 18 20 20	0 5 + 5 + 5 + 5 + 5 +	0 8 + 8 + 8 7 8 7 8 7	0 20 5 20 10 20 13 20 16 20 18	7 18 10 18 15 18 15 18 15 18 17	0 5 + 5 + 5 5 5 5 5 5	+ + + 5 + 5 + 8 + 8 +	0 5 + 5 + 5 + 5 + 5 +
	$x = -0.4 = 6$	$x = -5.6 = 5$	$x = -0.42 = 12$	$x = -0 = +$	$x = -0.33 = 15$	$x = -0 = 5$	$x = -0.14 = 7$	$x = -1.9 = 7$	$x = -0.2 = 15$	$x = -0 = 5$	$x = -0.6 = 5$	$x = -0 = 5$

TABLE 2.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS*-
SYMPTOMATICI AND ALLIED ORGANISMS *

Glyco- gen	Starch	Glyce- rin	Ado- nite	Sor- bite	Dul- cite	Man- nite	Rham- nose	Salic- in	Amyg- dalin	Phlori- zin	Carbohy- drate-free Broth
Bacillus anthracis-symptomatici											
0 5 + 5 5 5 5 5 5	0 13 8 15 12 15 12 15 13 15 13	0 8 + 8 5 8 5 8 5 8 5	+ 8 + 8 5 8 5 8 5 8 5	+ 20 12 20 13 23 15 23 16 23 20	0 7 + 7 5 7 5 7 5 7 5	0 10 0 10 5 10 7 10 10 10 10	+ 5 + 5 + 5 5 5 5 5 5	0 15 5 15 7 15 10 15 12 15 15	0 8 + 8 5 8 5 8 8 8 8	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0
$x = \frac{0}{5} = 0$	$x = \frac{2}{13} = 0.15$	$x = \frac{0}{8} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{8}{15} = 0.53$	$x = \frac{0}{7} = 0$	$x = \frac{0}{10} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{3}{12} = 0.25$	$x = \frac{0}{8} = 0$	$x = 0$	$x = 0$
+ 5 + 5 + 5 + 5 + 5 5	0 7 + 8 5 8 5 8 7 8 7	+ 10 7 10 8 10 8 10 10 10 10	+ 5 5 5 5 5 5 5 5 5 5	+ 12 + 13 5 15 5 12 9 12 10	0 8 + 8 5 8 5 8 5 8 5	0 15 8 15 13 15 14 15 15 15 15	+ 5 + 5 + 5 5 5 5 5 5	+ 19 8 19 12 19 15 20 15 20 15	0 5 + 8 5 8 5 5 5 5 5	0 0 0 0 0 + 0 + 0 + 0	0 0 0 0 0 0 0 + 0 + 0
$x = \frac{0}{5} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{2}{8} = 0.25$	$x = \frac{1}{4} = 0.25$	$x = \frac{4}{8} = 0.5$	$x = \frac{0}{8} = 0$	$x = \frac{5}{10} = 0.5$	$x = \frac{0}{5} = 0$	$x = \frac{4}{16} = 0.25$	$x = \frac{0}{5} = 0$	$x = \frac{0}{+} = 0$	$x = 0$
Bacillus oedematis-maligni, I											
0 + + + + + + + + 5 +	0 8 + 8 + 8 5 8 6 8 8	0 17 0 17 + 17 5 17 12 17 15	0 10 + 10 7 10 7 10 8 10 10	15 30 20 30 23 32 23 32 27 32 28	0 9 + 9 + 9 5 9 5 9 5	0 10 0 12 + 12 7 12 10 12 10	0 15 + 15 12 15 12 15 12 15 15	0 12 0 15 0 20 + 20 5 20 10	0 5 + 8 + 8 5 8 5 8 5	0 0 0 0 0 + 0 + 0 + 0	0 + 0 + + + + + + + +
$x = \frac{0}{5} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{3}{14} = 0.21$	$x = \frac{2}{8} = 0.25$	$x = \frac{12}{20} = 0.6$	$x = \frac{0}{9} = 0$	$x = \frac{0}{12} = 0$	$x = \frac{1}{14} = 0.07$	$x = \frac{3}{17} = 0.18$	$x = \frac{0}{8} = 0$	$x = \frac{0}{+} = 0$	$x = 0$
0 + + + + + + + + 5 +	+ 8 + 9 5 9 5 9 7 9 8	0 35 5 35 15 40 20 40 28 40 30	0 10 5 10 8 10 8 10 10 10 10	+ 10 + 10 5 13 5 10 8 10 10	0 10 5 10 7 10 5 8 8 10 8	0 8 0 8 5 8 5 8 7 8 7	+ 5 + 5 5 5 5 5 5 5 5	0 20 5 20 15 22 18 22 20 22 20	0 5 + 7 5 5 5 5 5 5 5	0 0 0 0 0 + 0 + 0 + 0	0 + 0 + 0 + + + + + +
$x = \frac{0}{5} = 0$	$x = \frac{2}{7} = 0.28$	$x = \frac{18}{22} = 0.82$	$x = \frac{0}{10} = 0$	$x = \frac{2}{8} = 0.25$	$x = \frac{0}{10} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{2}{20} = 0.1$	$x = \frac{0}{5} = 0$	$x = \frac{0}{+} = 0$	$x = 0$
+ 5 + 5 + 5 + 5 + 5 +	+ 13 10 13 12 13 12 13 13 13 13	0 10 + 10 + 18 5 18 5 18 5	+ 9 5 9 5 9 5 9 5 9 5	0 10 + 10 5 10 5 10 8 10 8	0 7 + 7 5 7 5 7 5 7 5	0 6 0 6 + 6 5 6 6 6 6	+ 5 + 5 + 5 + 5 5 5 5	0 27 10 27 20 27 25 27 27 27 27	+ 5 + 5 5 5 5 5 5 5 5	0 0 0 0 0 0 0 0 0 0 0	0 0 0 + 0 + 0 + 0 + 0 + 0
$x = \frac{0}{5} = 0$	$x = \frac{0}{13} = 0$	$x = \frac{8}{10} = 0.8$	$x = \frac{0}{9} = 0$	$x = \frac{0}{10} = 0$	$x = \frac{0}{7} = 0$	$x = \frac{0}{6} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{10}{17} = 0.58$	$x = \frac{0}{5} = 0$	$x = 0$	$x = 0$
0 + + + + + + + + + +	0 8 + 8 5 8 5 8 8 8 8	0 8 + 8 + 10 + 10 + 10 8	0 5 + 5 5 7 5 7 5 7 5	0 8 + 10 5 12 5 12 7 12 8	+ 7 + 7 5 7 5 7 5 7 5	0 5 0 5 + 9 5 9 5 9 5	+ 5 + 5 + 10 5 5 5 5 5	0 25 + 25 15 25 17 25 22 25 25	0 7 + 7 5 7 5 7 5 7 5	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0
$x = \frac{0}{3} = 0$	$x = \frac{1}{7} = 0.14$	$x = \frac{4}{6} = 0.67$	$x = \frac{1}{6} = 0.16$	$x = \frac{5}{7} = 0.7$	$x = \frac{0}{7} = 0$	$x = \frac{0}{9} = 0$	$x = \frac{0}{10} = 0$	$x = \frac{5}{20} = 0.25$	$x = \frac{0}{7} = 0$	$x = 0$	$x = 0$

TABLE 2.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS*-
SYMPTOMATIC AND ALLIED ORGANISMS *

Dex- trose	Levu- lose	Galac- tose	Arabi- nose	Man- nose	Xylose	Lac- tose	Mal- tose	Saccha- rose	Raffi- nose	Dex- trin	Inulin
<i>Bacillus oedematis-maligni</i> , II (Novy)											
+ 16 7 16 12 16 14 16 15 16 16	+ 28 5 30 10 30 17 32 23 32 28	+ 10 7 10 8 10 8 10 10 10 10	+ 8 7 8 7 10 7 10 8 10 8	+ 8 5 8 7 12 7 48 8 48 8	+ 5 + 5 + 5 + 5 + 5 5	+ 7 5 7 6 7 7 7 7 7 7	+ 32 10 32 20 37 27 37 32 37 32	+ 15 5 15 10 15 12 15 13 15 10	0 5 + 5 + 5 + 5 5 5 5	+ 5 + 5 + 5 5 5 5 5 5	+ 5 + 5 + 5 5 8 5 8 5
$x = -2.2$ 5	$x = -3$ 8	$x = -0.25$ 8	$x = -0.25$ 8	$x = -0.2$ 40	$x = -0.67$ 3	$x = -0$ 7	$x = -2.7$ 10	$x = -0.5$ 10	$x = -0$ 5	$x = -0$ 5	$x = -0$ 8
<i>B. botulinus</i>											
+ 5 + 5 + 7 + 7 + 7 5	0 11 + 11 5 11 7 14 8 14 8	0 + + + + + + + + + +	0 5 + 5 + 5 + 5 + 5 +	0 5 + 5 + 5 + 5 5 5 5	0 5 + 5 + 5 + 5 + 5 5	7 12 7 12 8 15 10 15 10 15 12	+ 13 5 13 8 13 10 13 12 13 13	+ 10 + 15 7 20 8 28 10 28 10	0 5 + 5 + 5 5 5 5 5 5	+ 5 + 5 + 10 + 10 + 10 +	0 + + + + 5 + 5 + 5 +
$x = -0.4$ 5	$x = -1.8$ 5	$x = -0$ +	$x = -0.25$ 4	$x = -0.25$ 4	$x = -0.67$ 3	$x = -0.15$ 13	$x = -3.33$ 3	$x = -1.33$ 12	$x = -0$ 5	$x = -0$ 10	$x = -0$ 5
<i>Ghon-Sachs bacillus</i>											
0 65 0 65 0 65 15 63 55 63 65	0 10 0 10 ? 13 + 13 5 13 7	0 25 0 25 0 30 + 30 10 30 17	0 10 + 10 5 10 5 100 7 100 7	0 0 0 0 0 0 0 15 0 15 0	0 8 + 8 5 8 5 8 8 8 8	0 20 0 20 7 20 10 20 12 20 15	0 12 0 12 ? 15 + 12 5 15 10	0 7 0 7 0 32 0 32 + 32 +	0 0 0 0 0 0 0 0 0 0 0	0 12 0 12 0 12 + 12 10 12 10	0 8 0 8 0 8 + 20 5 20 5
$x = -0.5$ 42	$x = -0$ 13	$x = -0.2$ 25	$x = -0$ 100	$x = -0.25$ 12	$x = -0$ 8	$x = -0$ 20	$x = -0$ 15	$x = -0$ 32	$x = 0$	$x = -0$ 12	$x = -0$ 20
<i>B. coli</i> (control)											
72 67 75 67 75 67 75 65 75 65 75	45 50 55 50 58 50 58 50 58 50 58	15 65 50 65 60 65 65 65 65 65 65	45 55 55 55 60 55 60 53 60 53 60	70 70 75 70 78 70 75 70 75 70 72	35 82 52 85 60 85 68 85 80 85 80	55 70 68 70 70 70 70 70 75 70 70	+ 80 40 80 70 80 75 80 75 80 75	30 63 53 63 65 63 70 65 70 65 72	50 87 70 87 78 87 82 87 82 87 82	0 32 0 32 + 35 5 32 10 32 30	0 0 0 0 0 0 0 0 0 0 0
$x = -0.3$ 50	$x = -0.43$ 35	$x = -0.44$ 45	$x = -0.33$ 40	$x = -0.5$ 47	$x = -1.13$ 40	$x = -0.55$ 45	$x = -0.6$ 50	$x = -0.44$ 45	$x = -1$ 43	$x = -0.07$ 30	$x = 0$

TABLE 2.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS*-
SYMPTOMATICI AND ALLIED ORGANISMS *

Glyco- gen	Starch	Glyc- erin	Ado- nite	Sor- bite	Dul- cite	Man- nite	Rham- nose	Sali- cin	Amyg- dalin	Phlori- zin	Carbohy- drate-free Broth
Bacillus oedematis-maligni, II (Novy)											
0 5 + 5 + 5 + 5 + 5	0 8 + 8 5 8 7 8 8 8	10 30 15 30 22 32 25 32 28 32 30	0 7 + 7 5 7 5 7 5 7 5	5 18 7 20 10 20 10 20 13 20 15	0 7 + 7 5 7 5 7 5 7 5	0 6 0 6 + 6 5 6 6 6 6	0 5 + 5 + 5 + 5 + 5 5	+ 12 5 12 7 12 8 12 10 12 10	+ 5 + 5 5 5 5 5 5 5 5	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0
$x = \frac{0}{5} = 0$	$x = \frac{1}{7} = 0.14$	$x = \frac{15}{17} = 0.9$	$x = \frac{0}{7} = 0$	$x = \frac{10}{10} = 1$	$x = \frac{0}{7} = 0$	$x = \frac{0}{6} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{2}{10} = 0.2$	$x = \frac{0}{5} = 0$	$x = 0$	$x = 0$
B. botulinus											
0 + + + + + + + + + +	0 + + + + + + + + + +	0 28 7 30 12 32 15 35 20 35 25	0 5 + 5 5 5 5 5 5 5 5	+ 18 5 21 7 22 10 22 12 22 15	0 6 + 6 5 6 5 6 5 6 5	0 5 0 5 + 5 + 5 + 5 5	0 5 + 5 + 5 + 5 + 5 5	+ 20 10 20 15 20 15 22 18 22 20	0 5 + 5 + 5 + 5 + 5 5	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0
$x = \frac{0}{3} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{18}{17} = 1.06$	$x = \frac{2}{3} = 0.67$	$x = \frac{14}{8} = 1.75$	$x = \frac{0}{6} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{5}{17} = 0.1$	$x = \frac{0}{5} = 0$	$x = 0$	$x = 0$
Ghon-Sachs bacillus											
+ 10 8 10 10 11 10 11 10 11 10	0 0 0 0 0 0 0 0 0 0 0	0 8 0 15 0 17 0 17 + 17 5	0 8 0 8 0 8 0 8 + 8 8	0 0 0 0 0 0 0 0 0 0 0	0 7 0 7 + 7 5 7 5 7 5	0 0 0 5 0 5 0 5 0 5 0	0 10 + 10 7 10 7 10 8 10 10	0 7 0 10 + 37 5 37 5 37 7	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0
$x = \frac{0}{11} = 0$	$x = 0$	$x = \frac{2}{15} = 0.13$	$x = \frac{0}{8} = 0$	$x = 0$	$x = \frac{0}{7} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{0}{10} = 0$	$x = \frac{2}{35} = 0.06$	$x = 0$	$x = 0$	$x = 0$
B. coli (control)											
0 + + + + + + + + + +	0 + + + + + + + + + +	0 0 0 0 0 0 0 0 0 0 0	0 5 + 5 + 5 + 5 5 5 5	52 83 82 80 90 80 92 70 88 70 83	0 11 + 11 5 12 8 12 10 12 10	37 70 52 70 60 75 65 75 70 70 70	27 57 47 60 50 60 55 60 57 60 57	0 55 + 55 47 55 55 55 55 55 55	0 5 + 5 + 8 + 8 5 8 5	0 + 0 + + + + + + 4 +	Negative through- out
$x = \frac{0}{4} = 0$	$x = \frac{0}{+} = 0$	$x = 0$	$x = \frac{0}{5} = 0$	$x = \frac{28}{42} = 0.67$	$x = \frac{6}{6} = 1$	$x = \frac{30}{40} = 0.75$	$x = \frac{30}{30} = 1$	$x = \frac{5}{50} = 0.1$	$x = \frac{2}{6} = 0.33$	$x = \frac{0}{4} = 0$	

TABLE 3

COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS*-
SYMPTOMATICI AND ALLIED ORGANISMS *

Strain	Dex- trose	Levu- lose	Galac- tose	Arabi- nose	Man- nose	Rham- nose	Xylose	Lac- tose	Mal- tose	Saceha- rose	Raffi- nose	Dex- trin
B. anthracis-symptomatici												
2	0 20	+ 62	0 +	0 5	0 13	0 5	0 10	0 5	0 38	0 +	0 +	0 5
	+ 22	25 65	+ +	+ 5	7 14	+ 5	+ 12	+ 5	10 42	+ +	0 +	0 5
	7 25	40 66	+ +	+ 5	11 14	+ 5	7 12	+ 5	18 45	+ +	+ 5	+ 5
	11 27	50 69	+ +	+ 5	12 14	+ 5	8 12	+ 5	24 48	+ +	+ 5	+ 5
	14 30	55 69	+ +	+ 5	12 14	5 5	10 12	+ 5	29 48	+ 5	+ 5	+ 5
	18	60	+	+	13	5	10	5	35	+	+	+
	$x = \frac{15}{15} = 1$	$x = \frac{49}{20} = 2.45$	$x = \frac{0}{+} = 0$	$x = \frac{1}{4} = 0.25$	$x = \frac{4}{10} = 0.4$	$x = \frac{0}{5} = 0$	$x = \frac{4}{8} = 0.5$	$x = \frac{0}{5} = 0$	$x = \frac{36}{12} = 3$	$x = \frac{0}{5} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{0}{5} = 0$
3	0 46	+ 48	0 5	0 5	8 22	0 8	0 7	0 5	0 50	0 10	0 7	0 +
	11 50	12 55	+ 5	+ 7	15 22	+ 8	+ 8	+ 5	11 50	0 10	0 7	0 +
	22 51	25 58	+ 5	+ 7	18 22	7 8	5 8	+ 5	25 50	+ 10	0 7	0 +
	32 52	33 63	+ 6	+ 8	20 22	7 8	5 8	+ 5	35 52	8 11	+ 7	+ +
	37 52	37 62	+ 6	5 8	21 22	8 8	7 8	5 5	40 50	10 11	5 7	+ +
	43	43	5	5	21	8	7	5	46	10	5	+
	$x = \frac{40}{12} = 3.33$	$x = \frac{50}{12} = 4.17$	$x = \frac{1}{5} = 0.2$	$x = \frac{4}{4} = 1$	$x = \frac{8}{14} = 0.57$	$x = \frac{0}{8} = 0$	$x = \frac{2}{6} = 0.33$	$x = \frac{0}{5} = 0$	$x = \frac{37}{13} = 2.85$	$x = \frac{2}{9} = 0.22$	$x = \frac{0}{7} = 0$	$x = \frac{0}{+} = 0$
4	+ 60	+ 38	0 +	0 6	0 9	0 10	0 6	0 7	15 62	0 11	0 +	0 7
	15 60	14 42	+ +	+ 6	+ 9	+ 10	+ 7	+ 7	28 63	+ 11	+ 5	0 7
	30 60	20 45	+ +	+ 6	5 9	9 10	+ 7	5 7	43 64	10 12	+ 5	+ 7
	40 60	27 49	+ +	5 6	7 9	9 10	5 7	5 7	54 65	10 12	+ 5	5 7
	50 60	30 50	+ +	5 6	8 9	10 10	6 7	7 7	58 65	10 12	+ 5	5 7
	57	35	+	5	8	10	6	7	60	11	+	5
	$x = \frac{40}{20} = 2$	$x = \frac{36}{14} = 2.57$	$x = \frac{0}{+} = 0$	$x = \frac{1}{5} = 0.2$	$x = \frac{4}{5} = 0.8$	$x = \frac{1}{9} = 0.11$	$x = \frac{2}{5} = 0.4$	$x = \frac{1}{6} = 0.17$	$x = \frac{43}{22} = 1.95$	$x = \frac{2}{10} = 0.2$	$x = \frac{1}{4} = 0.25$	$x = \frac{0}{7} = 0$
5	+ 32	+ 32	+ 16	0 5	0 10	0 8	0 7	0 7	0 45	0 15	+ 6	0 0
	15 32	18 33	13 17	+ 5	+ 10	+ 9	+ 8	+ 7	20 45	0 15	+ 6	0 0
	22 33	23 33	15 17	+ 5	7 10	7 9	+ 8	5 7	30 45	10 15	5 6	0 0
	27 33	28 34	15 17	+ 5	8 10	7 9	5 8	5 7	37 47	13 15	5 6	0 5
	50 33	30 34	15 17	+ 5	8 10	8 9	7 8	7 7	40 46	13 15	6 6	0 5
	31	30	15	5	8	8	7	7	42	13	6	0
	$x = \frac{23}{10} = 2.3$	$x = \frac{21}{13} = 1.61$	$x = \frac{4}{13} = 0.3$	$x = \frac{1}{4} = 0.25$	$x = \frac{2}{8} = 0.25$	$x = \frac{1}{8} = 0.13$	$x = \frac{2}{6} = 0.33$	$x = \frac{1}{6} = 0.17$	$x = \frac{28}{18} = 1.55$	$x = \frac{3}{12} = 0.25$	$x = \frac{1}{5} = 0.2$	$x = \frac{0}{5} = 0$
6	8 41	+ 75	+ 10	0 10	0 12	0 7	0 10	0 11	0 61	0 6	+ 8	0 5
	15 45	17 80	7 11	5 10	8 12	+ 8	+ 11	8 11	+ 61	+ 6	+ 8	+ 5
	21 50	30 80	8 11	8 10	10 13	+ 10	+ 11	9 11	22 61	+ 6	7 8	+ 5
	27 53	47 76	10 12	8 10	11 13	6 10	6 11	10 11	37 61	5 10	7 8	+ 5
	32 55	60 76	10 12	9 10	11 13	6 10	8 11	10 11	54 61	5 10	8 8	+ 5
	38	70	10	9	13	7	8	10	60	5	8	5
	$x = \frac{42}{13} = 3.2$	$x = \frac{54}{22} = 2.45$	$x = \frac{4}{8} = 0.5$	$x = \frac{1}{9} = 0.11$	$x = \frac{3}{10} = 0.3$	$x = \frac{0}{10} = 0$	$x = \frac{5}{6} = 0.83$	$x = \frac{1}{10} = 0.1$	$x = \frac{44}{17} = 2.6$	$x = \frac{3}{7} = 0.42$	$x = \frac{1}{7} = 0.14$	$x = \frac{0}{5} = 0$
10	0 28	0 48	+ 10	0 +	0 8	0 10	0 60	0 8	+ 84	0 14	+ 7	0 5
	10 30	11 52	5 10	+ 7	+ 9	+ 10	38 62	+ 8	30 84	+ 14	+ 7	0 5
	15 33	25 57	8 11	+ 7	7 9	7 10	47 62	7 8	58 81	11 14	6 7	+ 5
	20 34	37 59	9 11	+ 7	8 9	9 10	52 62	8 8	72 81	13 15	6 7	+ 5
	25 35	42 63	9 11	+ 7	8 9	9 10	56 62	8 8	80 77	14 15	7 7	+ 5
	35	63	11	7	9	10	60	8	84	14	7	+
	$x = \frac{24}{11} = 2.18$	$x = \frac{50}{13} = 3.84$	$x = \frac{4}{7} = 0.56$	$x = \frac{1}{6} = 0.17$	$x = \frac{2}{7} = 0.28$	$x = \frac{0}{10} = 0$	$x = \frac{28}{34} = 0.82$	$x = \frac{1}{7} = 0.14$	$x = \frac{55}{22} = 2.5$	$x = \frac{3}{12} = 0.25$	$x = \frac{0}{7} = 0$	$x = \frac{0}{5} = 0$
17	0 7	0 11	+ 7	0 5	0 11	0 10	0 8	0 8	10 55	0 10	+ 10	0 10
	+ 8	+ 13	5 8	+ 5	7 12	+ 10	+ 8	+ 8	37 55	0 11	8 10	0 10
	5 8	5 13	6 8	+ 5	10 12	7 10	+ 9	7 8	46 55	0 11	10 10	+ 10
	6 9	8 13	7 8	+ 5	11 12	8 10	7 9	8 8	53 55	0 11	10 10	8 10
	6 10	10 14	7 8	+ 5	11 13	8 10	7 9	8 8	55 51	10 11	10 10	10 10
	10	14	8	5	12	10	8	8	55	10 11	10	10
	$x = \frac{5}{5} = 1$	$x = \frac{8}{6} = 1.25$	$x = \frac{2}{6} = 0.33$	$x = \frac{2}{3} = 0.67$	$x = \frac{3}{9} = 0.33$	$x = \frac{2}{8} = 0.25$	$x = \frac{2}{7} = 0.28$	$x = \frac{0}{8} = 0$	$x = \frac{33}{18} = 1.83$	$x = \frac{0}{11} = 0$	$x = \frac{1}{9} = 0.11$	$x = \frac{1}{9} = 0.11$

* Readings made every 24 hours for 11 days. Amount of gas produced indicated in percentages. Less than 5% is designated +.
 $x = \text{CO}_2/\text{H}_2$.
 Blank control negative throughout.

TABLE 3.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY BACILLUS ANTHRACIS-
SYMPTOMATICI AND ALLIED ORGANISMS *

Inulin	Glyco- gen	Starch	Glyce- rin	Ado- nite	Sor- bite	Dulcite	Man- nite	Salic- in	Amyg- dalin	Phlori- zin	Plain Carbohy- drate-free Broth
B. anthracis-symptomatici											
0 5 0 5 + 5 + 5 5	0 0 0 0 0 0 0 0 0	0 6 0 6 0 6 + 6 6	0 10 0 10 + 10 9 10 10	0 5 0 5 0 5 + 5 5	0 30 56 0 37 11 42 16 47 19 50 25 53	0 5 0 7 + 7 + 7 5	0 14 0 15 10 15 12 15 13 15 14	0 8 13 0 10 0 10 + 10 6 11 8 12	0 9 0 10 0 10 + 10 7 10 9	0 0 0 0 0 0 0 0 0 0 0	0 10 0 10 7 10 9 10 10 10 10
$x = -0$ 5	$x = -0$ +	$x = -0$ 6	$x = -0$ 10	$x = -0$ 5	$x = -1.67$ 21	$x = -0$ 7	$x = -0.15$ 13	$x = -0.3$ 10	$x = -0$ 10	$x = 0$	$x = -0$ 10
0 + 0 + 0 + + + + + +	0 + 0 + + 5 + 5 +	0 0 0 0 0 0 0 0 0 0 0	0 8 0 8 + 8 7 8 7 8 8	0 8 0 8 0 8 + 9 6 9 8	0 11 15 + 13 7 13 8 14 10 15 11 15	0 7 0 7 + 7 5 7 7 7 7	0 11 0 11 + 12 9 12 10 12 10	0 + 12 0 6 0 8 0 8 0 10 + 12	0 5 0 7 0 7 + 7 + 7 5	0 0 0 0 0 0 0 0 0 0 0	0 7 0 9 0 9 0 9 0 9 +
$x = -0$ +	$x = -0$ 5	$x = 0$	$x = -0$ 8	$x = -0$ 9	$x = -0.36$ 11	$x = -0$ 7	$x = -0.2$ 10	$x = -0.2$ 10	$x = -0$ 7	$x = 0$	$x = -0$ 9
0 + + + + + + + + + +	0 5 + 5 + 5 + 5 +	0 0 0 0 0 0 0 0 0 0 0	0 11 0 11 + 11 9 11 10 11 11	0 10 + 10 7 10 8 11 8 11 10	0 14 18 0 16 7 16 10 17 11 18 14 18	0 10 0 12 7 12 9 12 10 12 10	0 13 + 13 10 13 11 13 12 13 12	0 13 22 0 14 0 15 0 17 + 20 12 22	0 5 0 5 0 5 + 5 + 5 +	0 0 0 0 0 0 0 0 0 0 0	0 10 0 10 0 10 + 10 6 10 10
$x = -0$ +	$x = -0.4$ 3	$x = 0$	$x = -0$ 11	$x = -0.38$ 8	$x = -0.29$ 14	$x = -0.2$ 10	$x = -0.18$ 11	$x = -0.22$ 18	$x = -0$ 5	$x = 0$	$x = -0$ 10
0 + 0 5 + 5 + 5 +	0 0 0 + 0 + 0 + 0	0 8 + 8 7 8 7 8 8	0 13 0 13 0 13 12 13 13 13	0 12 + 12 10 12 10 12 10 12 12	0 12 14 + 12 7 12 9 12 9 13 12 14	0 7 0 7 + 7 5 7 7 7 7	0 15 0 15 7 15 13 15 14 15 14	0 0 13 0 5 0 5 0 11 0 12 0 13	0 7 0 8 0 8 + 8 + 8 7	0 0 0 0 0 0 0 0 0 0 0	0 0 0 7 0 10 0 10 0 10 0
$x = -0$ 5	$x = -0$ +	$x = -0$ 8	$x = -0$ 13	$x = -0.09$ 11	$x = -0.4$ 10	$x = -0$ 7	$x = -0.07$ 14	$x = -0$ 13	$x = -0$ 8	$x = 0$	$x = -0$ 10
0 + + + + + + + + + +	0 7 + 7 5 7 6 7 7 7 7	0 17 + 17 14 17 14 17 15 17 17	0 9 0 9 0 9 0 9 0 9 0	0 8 0 8 0 8 5 8 7 8 8	0 20 33 0 23 10 25 15 28 16 31 18 32	0 8 0 10 7 10 7 10 8 10 8	0 11 0 11 0 12 0 12 6 12 11	0 0 5 0 0 0 0 0 + 0 + 0 5	0 + 0 5 0 5 + 5 + 5 +	0 0 0 0 0 0 0 0 0 0 0	+ 14 5 14 13 14 14 14 14 14 14
$x = -0$ +	$x = -0.17$ 6	$x = -0.13$ 15	$x = -0$ 9	$x = -0.14$ 7	$x = -0.65$ 20	$x = -0.11$ 9	$x = -0$ 12	$x = -0$ 5	$x = -0$ 5	$x = 0$	$x = -0$ 14
0 + 0 + + + + + + + +	0 + 0 + + 8 + 8 + 5	0 8 + 8 + 8 7 8 8 8 8	0 10 0 10 0 11 0 11 0 11 8	0 6 0 6 + 6 + 6 5 6 6	0 25 35 0 30 12 30 15 32 18 33 23 34	0 9 0 10 7 10 8 10 9 10 9	10 76 85 76 83 75 78 72 75 72 78	0 35 42 0 38 30 40 33 40 33 41 35 42	0 + 0 + 0 + + 5 + 5 +	0 0 0 0 0 0 0 0 0 0 0	0 13 0 13 + 13 8 13 11 13 13
$x = -0$ +	$x = -0$ 5	$x = -0.14$ 7	$x = -0$ 11	$x = -0$ 6	$x = -0.67$ 21	$x = -0.11$ 9	$x = -0.36$ 53	$x = -0.82$ 23	$x = -0$ 5	$x = 0$	$x = -0$ 13
0 0 0 + 0 + 0 + 0 + 0	0 + 0 + 0 + 0 + +	0 0 0 0 0 0 0 0 0 0 0	0 8 0 9 0 9 0 9 8 9 8	0 11 0 11 8 11 8 11 10 11 11	0 25 31 0 28 15 28 17 30 19 31 23 31	0 5 0 6 + 6 + 6 5 6 5	0 8 0 8 + 8 7 8 8 8 8	0 23 27 0 25 0 25 + 25 + 25 20 27	0 6 0 6 0 6 + 6 + 6 +	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0
$x = -0$ +	$x = -0$ +	$x = 0$	$x = -0$ 9	$x = -0.1$ 10	$x = -0.4$ 22	$x = -0$ 6	$x = -0$ 8	$x = -0.17$ 23	$x = -0$ 6	$x = 0$	$x = 0$

TABLE 3.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS*-
SYMPTOMATIC AND ALLIED ORGANISMS *

Strain	Dextrose	Levulose	Galactose	Arabinose	Mannose	Rhamnose	Xylose	Lactose	Maltose	Saccharose	Raffinose	Dextrin
B. anthracis-symptomatici												
48	+ 7 28 11 35 15 40 18 41 25 41	0 24 + 26 8 26 14 28 18 28 21	+ 9 9 5 9 6 10 8 10 8 10 10	0 5 5 + 5 5 + 5 5 + 5 5 + 5 5 5 5	0 10 10 + 10 10 5 12 12 8 12 12 8 12 9	0 7 7 + 7 7 5 7 7 7 7 7 7 7 7 7	0 19 19 10 20 13 20 15 20 17 20 19	0 5 5 + 5 5 + 5 5 5 5 5 5 5	+ 43 43 10 47 15 50 23 54 28 54 37	0 8 8 + 8 8 6 8 8 6 8 8 6 8 8 6	0 11 11 0 11 11 0 11 11 0 11 11 0 11 11 10	0 8 8 + 8 8 7 8 8 7 8 8 7 8 8 7
	$x = \frac{32}{9} = 3.55$	$x = \frac{20}{8} = 2.5$	$x = \frac{1}{9} = 0.11$	$x = \frac{2}{3} = 0.67$	$x = \frac{3}{9} = 0.33$	$x = \frac{0}{7} = 0$	$x = \frac{7}{13} = 0.53$	$x = \frac{0}{5} = 0$	$x = \frac{41}{13} = 3.15$	$x = \frac{1}{7} = 0.14$	$x = \frac{1}{10} = 0.1$	$x = \frac{1}{7} = 0.14$
60	+ 8 43 13 48 18 53 25 53 35	10 74 15 75 28 73 43 73 53 70 67	+ 8 8 5 8 8 6 8 8 7 8 8 7 8 8	0 + + + + + + + + + + 5 + 5	0 7 7 + 7 7 + 7 7 5 7 7 5 7 6	0 5 5 + 6 6 + 6 6 5 6 6 5 6 5	0 10 10 + 10 10 5 10 10 10 9 10 9	0 5 5 + 7 7 + 7 7 + 7 7 + 7 +	5 75 14 77 35 77 54 77 65 75 72	0 11 11 0 11 11 8 11 10 11 10 11 11	+ 5 5 + 5 5 + 5 5 + 5 + +	0 + + 0 + + + + + + + + + + +
	$x = \frac{43}{10} = 4.3$	$x = \frac{58}{12} = 4.8$	$x = \frac{3}{5} = 0.6$	$x = \frac{1}{4} = 0.25$	$x = \frac{1}{6} = 0.17$	$x = \frac{1}{5} = 0.2$	$x = \frac{3}{7} = 0.42$	$x = \frac{0}{7} = 0$	$x = \frac{57}{18} = 3.16$	$x = \frac{2}{9} = 0.22$	$x = \frac{1}{4} = 0.25$	$x = \frac{0}{+} = 0$
Ghon-Sachs bacillus I												
	0 12 0 17 + 20 + 23 7 24 24	0 + 5 0 7 + 10 + 10 +	0 15 15 0 18 18 0 21 21 + 25 25 7 28 12	0 5 5 0 5 5 + 6 6 + 6 6 5	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	0 33 33 0 33 33 25 33 30 32 32 32 33	0 + + 0 + + 0 + + 0 + + 0 + + 0	0 0 0 0 0 0 0 9 9 0 13 13 0 15 15 0	0 20 20 + 20 20 5 20 8 24 13 24 16	0 8 8 0 8 8 + 8 8 + 8 8 7 8 7	0 10 10 0 10 10 + 10 10 7 10 10 8 10 10 8	0 + + 0 + + 0 5 5 0 5 5 0 5 5 +
	$x = \frac{8}{16} = 0.5$	$x = \frac{3}{7} = 0.42$	$x = \frac{8}{20} = 0.4$	$x = \frac{0}{6} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{12}{20} = 0.6$	$x = \frac{0}{+} = 0$	$x = \frac{2}{13} = 0.15$	$x = \frac{6}{18} = 0.33$	$x = \frac{0}{8} = 0$	$x = \frac{0}{10} = 0$	$x = \frac{0}{5} = 0$
B. oedematis-maligni, I												
421	10 28 15 30 18 30 21 31 24 31 25	0 14 + 15 8 15 11 15 12 15 13	+ 10 10 6 10 10 7 10 10 8 10 10 8 10 8	0 5 5 + 5 5 + 5 5 + 5 5 + 5 5 +	+ 13 13 + 13 13 7 13 13 9 13 13 10 13 13 12	0 7 7 + 7 7 + 7 7 5 7 7 7 7 7 7	0 10 10 + 10 10 + 11 11 6 11 11 7 11 11 7	+ 7 7 5 7 7 6 7 7 6 7 7 6 7 7 6	35 71 60 71 70 69 75 69 73 65 71	0 13 13 + 14 14 9 14 14 10 15 15 12 15 15 13	+ 8 8 5 8 8 7 8 8 7 8 8 7 8 7	0 5 5 + 5 5 + 5 5 + 5 5 + 5 5 5
	$x = \frac{21}{10} = 2.1$	$x = \frac{10}{5} = 2$	$x = \frac{3}{7} = 0.42$	$x = \frac{1}{4} = 0.25$	$x = \frac{4}{9} = 0.44$	$x = \frac{1}{6} = 0.17$	$x = \frac{4}{7} = 0.57$	$x = \frac{1}{6} = 0.17$	$x = \frac{13}{52} = 0.25$	$x = \frac{4}{11} = 0.36$	$x = \frac{1}{7} = 0.14$	$x = \frac{0}{5} = 0$
485	+ 12 22 12 23 15 23 17 24 18 24 20	0 19 + 20 10 20 14 20 16 20 18	+ 10 10 7 10 10 8 11 10 11 10 11 10	0 8 8 + 8 8 5 8 8 7 8 8 7 8 7	0 13 13 8 13 13 10 13 13 12 13 13 12 13 13 13	0 11 11 7 11 11 9 11 11 9 11 11 10 11 11 11	0 13 13 7 13 13 8 13 13 10 13 13 12 13 13 12	+ 10 10 8 10 10 9 10 10 10 10 10 10 10 10 10	+ 55 55 35 55 42 55 57 55 56 50 56	0 13 13 + 13 13 9 13 13 10 13 13 11 13 13 11	0 14 14 0 14 14 0 14 14 8 15 15 12 15 15 13	0 + + 0 + + + + + + + + + + + +
	$x = \frac{14}{10} = 1.4$	$x = \frac{11}{9} = 1.2$	$x = \frac{2}{9} = 0.22$	$x = \frac{1}{7} = 0.14$	$x = \frac{2}{11} = 0.18$	$x = \frac{1}{10} = 0.1$	$x = \frac{4}{9} = 0.44$	$x = \frac{0}{10} = 0$	$x = \frac{33}{17} = 1.94$	$x = \frac{3}{10} = 0.3$	$x = \frac{2}{13} = 0.15$	$x = \frac{0}{+} = 0$
558	+ 8 17 18 18 10 20 13 20 14 20 15	0 12 + 13 5 13 8 14 9 14 10	0 5 5 + 5 5 + 6 6 5 6 6 5 6 6 5	0 + + + 5 5 + 5 5 + 5 5 + 5 5 +	0 15 15 11 15 15 13 15 15 14 15 15 14 15 15 15	+ 12 12 10 12 12 10 12 12 11 12 12 11 12 12 11	0 7 7 + 8 8 + 8 8 6 8 8 7 8 8 7	0 8 8 5 8 8 6 8 8 7 8 8 7 8 7	13 72 42 71 58 71 68 71 70 69 70	0 12 12 + 13 13 10 13 13 10 13 13 10 13 13 12	+ 10 10 + 10 10 + 10 10 10 10 10 10 10 10 10	+ 6 6 + 6 6 + 6 6 5 6 6 5 6 6 6
	$x = \frac{12}{8} = 1.5$	$x = \frac{9}{5} = 1.8$	$x = \frac{0}{6} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{3}{12} = 0.25$	$x = \frac{0}{12} = 0$	$x = \frac{2}{6} = 0.33$	$x = \frac{1}{7} = 0.14$	$x = \frac{51}{18} = 2.83$	$x = \frac{2}{11} = 0.18$	$x = \frac{0}{10} = 0$	$x = \frac{0}{6} = 0$

TABLE 3.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS*-
SYMPTOMATICI AND ALLIED ORGANISMS *

Inulin	Glyco- gen	Starch	Glyce- rin	Ado- nite	Sor- bite	Dulcite	Man- nite	Salic- in	Amyg- dalin	Phlori- zin	Plain Carbohy- drate-free Broth
B. anthracis-symptomatici											
0 6 + 7 6 6 6 7 6 7	0 8 0 8 0 8 5 8 7 8	0 8 0 8 + 8 7 8 8 8	0 8 0 8 0 8 + 8 7 8 8	+ 6 + 6 5 6 5 6 5 6 6	+ 43 68 12 51 14 57 15 62 18 64 35 66	0 9 0 10 8 10 8 10 9 10	0 8 + 8 + 8 7 8 7 8	0 17 25 0 20 0 21 + 21 10 23 16 25	0 10 0 12 0 12 6 12 9 12	0 + 0 + 0 + 0 + + + +	0 10 0 12 0 12 0 12 8 12 10
$x = \frac{0}{7} = 0$	$x = \frac{4}{4} = 1$	$x = \frac{0}{8} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{0}{6} = 0$	$x = \frac{47}{21} = 2.24$	$x = \frac{1}{9} = 0.11$	$x = \frac{0}{8} = 0$	$x = \frac{5}{20} = 0.25$	$x = \frac{0}{12} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{12} = 0$
0 4 0 4 + 4 + 4 + 4	0 + 0 + 0 + 0 + +	0 7 0 8 + 8 5 8 7 8	0 10 0 10 0 10 + 10 9 10 10	0 + 0 + + 6 + 6 + 6 +	0 18 26 0 20 10 22 12 23 13 25 16 26	0 6 0 6 + 6 + 6 + 6 6	0 10 0 12 + 12 10 12 10 12 10	0 0 5 0 0 0 0 0 0 0 + 0 5	0 5 0 5 0 7 + 7 + 7 5 7	0 0 0 0 0 + 0 + 0 + 0	0 7 0 7 0 7 + 7 5 7 5
$x = \frac{0}{4} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{0}{10} = 0$	$x = \frac{0}{6} = 0$	$x = \frac{8}{18} = 0.44$	$x = \frac{0}{6} = 0$	$x = \frac{1}{11} = 0.09$	$x = \frac{0}{5} = 0$	$x = \frac{0}{7} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{7} = 0$
Ghon-Sachs bacillus I											
0 9 0 10 + 10 7 10 7 10 9	0 10 0 10 0 10 + 10 9 10 9	0 + 0 + 0 + + + + + +	0 + 0 + 0 + + + + + +	0 12 0 12 0 12 + 12 11 12 12	0 0 0 0 0 0 0 0 0 0 0 0	0 92 70 92 77 92 82 87 85 87 92	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0 0	0 0 0 + 0 + 0 + 0 + 0 + 0	0 0 0 0 0 + 0 + 0 + 0	0 0 0 0 0 0 0 0 0 0 0
$x = \frac{0}{10} = 0$	$x = \frac{0}{10} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{12} = 0$	$x = 0$	$x = \frac{27}{60} = 0.45$	$x = 0$	$x = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{+} = 0$	$x = 0$
B. oedematis-maligni, I											
0 + 0 5 + 5 + 5 +	0 7 + 7 + 7 5 7 5	0 17 15 17 17 17 17 17 17	0 + 0 8 0 8 0 8 0	0 11 0 11 7 11 9 11 9 11 11	0 12 24 0 15 0 16 0 18 6 20 11 22	0 16 0 17 8 17 13 17 14 17 16	0 7 0 8 0 8 + 8 + 8 +	0 11 19 0 13 0 15 0 15 + 17 9 19	0 5 0 6 0 6 + 6 + 6 +	0 0 0 0 0 0 0 0 0 0 0	0 15 0 15 0 15 + 15 13 15 15
$x = \frac{0}{5} = 0$	$x = \frac{1}{6} = 0.17$	$x = \frac{1}{16} = 0.06$	$x = \frac{0}{8} = 0$	$x = \frac{1}{10} = 0.1$	$x = \frac{6}{18} = 0.33$	$x = \frac{2}{15} = 0.13$	$x = \frac{0}{8} = 0$	$x = \frac{0}{19} = 0$	$x = \frac{0}{6} = 0$	$x = 0$	$x = \frac{0}{15} = 0$
0 5 0 7 5 7 5 7 5	0 5 0 5 0 5 0 5 +	0 5 0 5 0 5 + 5 5	0 25 0 28 0 28 14 28 20 27 24	0 8 + 8 7 8 7 8 7 8 8	0 43 60 0 51 18 55 23 57 25 59 33 60	+ 11 10 12 10 12 10 12 10 12 11	0 10 + 12 + 12 8 12 9 12 10	0 19 31 0 23 0 25 + 25 9 27 15 29	0 11 0 11 + 12 8 12 10 12 11	0 0 0 0 0 + 0 + 0 + 0	0 10 0 11 0 11 8 11 10 11 10
$x = \frac{0}{7} = 0$	$x = \frac{1}{4} = 0.25$	$x = \frac{0}{5} = 0$	$x = \frac{11}{16} = 0.69$	$x = \frac{1}{7} = 0.14$	$x = \frac{32}{28} = 1.14$	$x = \frac{2}{10} = 0.2$	$x = \frac{2}{10} = 0.2$	$x = \frac{3}{28} = 0.11$	$x = \frac{0}{12} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{11} = 0$
0 + 0 + + + + + +	0 + 0 + + 7 +	0 0 0 0 0 0 0 0 0	0 8 0 9 + 9 6 9 6 9 8	0 + 0 5 0 5 0 5 +	8 21 26 18 22 19 23 19 23 19 25 20 26	0 11 + 11 10 11 10 11 10 11 11	0 7 0 8 + 8 5 8 7	0 30 33 + 32 12 32 25 32 26 32 29 33	0 10 0 10 0 10 + 10 8 10 8	0 0 0 0 0 0 0 0 0 0 0	0 13 0 13 8 13 13 13 13 13 13
$x = \frac{0}{+} = 0$	$x = \frac{1}{6} = 0.17$	$x = 0$	$x = \frac{0}{9} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{8}{18} = 0.44$	$x = \frac{1}{10} = 0.1$	$x = \frac{0}{8} = 0$	$x = \frac{8}{25} = 0.32$	$x = \frac{0}{10} = 0$	$x = 0$	$x = \frac{0}{13} = 0$

TABLE 3.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS*-
SYMPTOMATICI AND ALLIED ORGANISMS *

Strain	Dex- trose	Levu- lose	Galac- tose	Arabi- nose	Man- nose	Rham- nose	Xylose	Lac- tose	Mal- tose	Saccha- rose	Raffi- nose	Dex- trin
B. oedematis-maligni, I												
560	0 7	0 17	0 +	0 +	0 11	0 8	0 7	0 10	+ 18	0 23	+ 10	0 +
	+ 7	+ 18	+ +	+ +	+ 13	+ 8	+ 7	7 10	10 18	+ 24	+ 10	+ +
	+ 8	5 18	+ +	+ 4	5 13	+ 8	+ 7	10 10	13 19	10 25	9 10	+ +
	5 8	10 20	+ +	+ 4	7 14	7 8	+ 7	10 13	15 19	13 28	10 10	+ +
	5 8	13 20	+ +	+ 4	8 14	8 8	5 7	10 13	15 19	17 28	10 10	+ +
	6	15	+	+	10	8	5	10	17	20	10	+
	$x = \frac{5}{3} = 1.67$	$x = \frac{15}{5} = 3$	$x = \frac{0}{+} = 0$	$x = \frac{1}{3} = 0.33$	$x = \frac{4}{10} = 0.4$	$x = \frac{0}{8} = 0$	$x = \frac{1}{6} = 0.17$	$x = \frac{3}{10} = 0.3$	$x = \frac{14}{5} = 2.8$	$x = \frac{10}{18} = 0.55$	$x = \frac{2}{8} = 0.25$	$x = \frac{0}{+} = 0$
B. oedematis-maligni, II, (Novy 1-ME II)												
	0 15	0 12	0 8	0 5	0 0	0 8	0 6	0 6	0 26	0 +	0 6	0 5
	+ 16	+ 14	+ 8	+ 5	0 4	0 8	0 8	+ 6	8 26	+ 5	+ 6	0 5
	8 16	+ 15	6 8	+ 5	0 4	+ 8	+ 8	5 6	15 27	+ 5	+ 6	+ 5
	11 16	7 16	7 8	+ 5	0 4	+ 8	5 8	5 6	20 27	+ 5	+ 6	+ 5
	13 16	7 16	7 8	5 5	0 4	7 8	6 8	6 6	23 27	+ 5	+ 6	+ 5
	15	10	7	5	0	8	6	6	25	+	+	5
	$x = \frac{12}{4} = 3$	$x = \frac{9}{7} = 1.3$	$x = \frac{2}{6} = 0.33$	$x = \frac{2}{3} = 0.67$	$x = \frac{1}{3} = 0.33$	$x = \frac{0}{8} = 0$	$x = \frac{2}{6} = 0.33$	$x = \frac{0}{6} = 0$	$x = \frac{21}{6} = 3.5$	$x = \frac{1}{4} = 0.25$	$x = \frac{1}{5} = 0.2$	$x = \frac{0}{5} = 0$
B. botulinus												
	0 13	0 12	0 5	0 5	0 6	0 5	0 8	0 7	0 15	0 10	+ 5	0 7
	5 14	+ 14	+ 5	+ 5	+ 6	+ 5	+ 8	+ 7	+ 17	+ 10	+ 5	0 7
	8 14	+ 14	+ 5	+ 5	+ 6	+ 5	+ 8	+ 7	8 19	6 10	5 5	+ 7
	10 14	5 15	+ 5	+ 6	5 6	+ 5	5 8	5 7	10 19	7 10	5 5	+ 7
	10 14	8 15	5 5	+ 6	5 6	+ 5	7 7	5	12 19	8 10	5 5	6 7
	12	10	5	5	5	5	7	5	13	8	5	6
	$x = \frac{9}{5} = 1.8$	$x = \frac{10}{5} = 2$	$x = \frac{1}{4} = 0.25$	$x = \frac{0}{6} = 0$	$x = \frac{0}{6} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{2}{6} = 0.33$	$x = \frac{1}{6} = 0.17$	$x = \frac{11}{8} = 1.37$	$x = \frac{2}{8} = 0.25$	$x = \frac{0}{5} = 0$	$x = \frac{0}{7} = 0$
B. coli (control)												
	33 55	67 70	8 61	60 78	70 68	20 50	37 75	65 77	28 83	45 88	42 90	0 5
	48 56	70 71	30 65	72 77	71 68	40 52	65 80	73 77	63 80	62 90	75 90	+ 5
	51 58	71 71	38 67	78 75	71 68	45 52	70 77	76 75	70 78	75 90	90 88	+ 5
	55 60	73 72	48 70	80 75	72 68	48 50	73 73	78 75	83 75	85 90	90 88	+ 5
	55 63	70 72	53 72	78 72	70 68	48 50	72 73	77 75	87 73	88 90	90 88	+ 5
	55	70	58	78	68	50	75	77	87	88	90	5
	$x = \frac{30}{33} = 0.90$	$x = \frac{30}{42} = 0.71$	$x = \frac{44}{28} = 1.57$	$x = \frac{27}{45} = 0.6$	$x = \frac{28}{40} = 0.7$	$x = \frac{20}{30} = 0.67$	$x = \frac{26}{47} = 0.55$	$x = \frac{35}{40} = 0.9$	$x = \frac{41}{32} = 1.28$	$x = \frac{48}{42} = 1.14$	$x = \frac{48}{40} = 1.2$	$x = \frac{0}{5} = 0$

TABLE 3.—Continued
COMPARISON OF RESULTS IN GAS-PRODUCTION FROM SUGARS BY *BACILLUS ANTHRACIS*-
SYMPTOMATICI AND ALLIED ORGANISMS *

Inulin	Glyco- gen	Starch	Glyce- rin	Ado- nite	Sor- bite	Dulcite	Man- nite	Sali- cin	Amyg- dalin	Phlori- zin	Plain Carbohy- drate-free Broth
B. oedematis-maligni, I											
0 6 0 7 + 7 + 7 + 7 + 6	0 7 + 7 + 7 + 7 + 7 +	0 8 + 8 5 8 5 8 8 8 8	0 32 + 34 20 34 25 37 28 37 30	0 5 0 5 + 5 + 5 + 5 5	0 14 20 + 17 6 17 10 17 12 19 14 20	0 5 0 5 + 5 + 5 + 5 5	0 7 0 9 + 9 + 9 7 9 7	0 5 8 0 5 0 5 + 7 + 8 5 8	0 5 0 7 0 7 + 7 + 7 +	0 0 0 0 0 + 0 + 0 + 0	0 8 0 8 + 8 + 8 + 8 6
$x = \frac{1}{6} = 0.17$	$x = \frac{0}{7} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{11}{26} = 0.42$	$x = \frac{0}{5} = 0$	$x = \frac{8}{12} = 0.67$	$x = \frac{0}{5} = 0$	$x = \frac{0}{9} = 0$	$x = \frac{0}{8} = 0$	$x = \frac{0}{7} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{8} = 0$
B. oedematis-maligni, II, (Novy 1-ME II)											
0 7 0 7 + 7 + 7 + 7 7	0 + 0 + 0 + + + + + +	0 7 5 7 5 7 7 7 7 7 7	0 30 0 35 + 40 13 43 19 43 24	0 7 0 7 0 7 + 7 + 7 7	0 12 15 + 13 8 13 9 13 10 14 12 15	0 8 0 8 + 9 5 9 8 9 8	0 10 0 10 + 10 7 10 8 10 8	0 5 10 0 8 0 8 + 8 + 9 5 10	0 9 0 10 0 10 + 10 5 10 9	0 0 0 0 0 0 0 0 0 0 0	0 6 0 7 + 7 + 7 + 7 6
$x = \frac{0}{7} = 0$	$x = \frac{0}{+} = 0$	$x = \frac{0}{7} = 0$	$x = \frac{16}{27} = 0.6$	$x = \frac{0}{7} = 0$	$x = \frac{5}{10} = 0.5$	$x = \frac{0}{9} = 0$	$x = \frac{1}{9} = 0.11$	$x = \frac{0}{10} = 0$	$x = \frac{0}{10} = 0$	$x = 0$	$x = \frac{0}{7} = 0$
B. botulinus											
0 + 0 + + + + + + + +	0 + + + + + + + + + +	0 0 0 0 0 0 0 0 0 0 0	0 22 0 23 10 25 15 28 17 28 20	0 + 0 6 0 6 + 6 + 6 +	0 13 23 + 15 8 17 10 19 11 21 13 23	0 5 0 6 + 6 + 6 5 6 5	0 10 0 10 + 10 8 10 9 10 9	0 5 8 0 7 0 7 + 7 + 8 + 8	0 8 0 9 0 9 + 9 5 9 7	0 0 0 0 0 0 0 0 0 0 0	0 10 0 10 + 10 5 10 8 10 8
$x = \frac{0}{+} = 0$	$x = \frac{0}{+} = 0$	$x = 0$	$x = \frac{10}{18} = 0.55$	$x = \frac{0}{6} = 0$	$x = \frac{11}{12} = 0.92$	$x = \frac{0}{6} = 0$	$x = \frac{1}{9} = 0.11$	$x = \frac{0}{8} = 0$	$x = \frac{0}{9} = 0$	$x = 0$	$x = \frac{0}{10} = 0$
B. coli (control)											
0 + 0 + + + + + + + +	0 5 + 5 + 5 + 5 + 5 +	0 18 0 38 0 47 0 50 + 50 7	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0	64 83 90 84 90 81 87 77 82 77 83	0 95 65 95 90 95 92 90 92 90 95	50 73 75 75 76 73 75 73 71 73 73	0 51 0 52 35 52 48 50 48 50 51	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0	0 7 0 7 + 7 5 7 7 7 7
$x = \frac{0}{+} = 0$	$x = \frac{0}{5} = 0$	$x = \frac{11}{39} = 0.26$	$x = 0$	$x = 0$	$x = \frac{20}{57} = 0.35$	$x = \frac{25}{65} = 0.38$	$x = \frac{23}{50} = 1.46$	$x = \frac{15}{35} = 0.42$	$x = 0$	$x = 0$	$x = \frac{0}{7} = 0$

TABLE 4
 SUMMARIZED READINGS TAKEN FROM TABLE 3

		Dextrose		Levulose		Mannose		Maltose		Saccharose	
		CO ₂	H	CO ₂	H	CO ₂	H	CO ₂	H	CO ₂	H
B. anthracis-symptomatici..	2	15	15	49	20	4	10	36	12	0	5
	3	40	12	50	12	8	14	37	13	2	9
	4	40	20	36	14	4	5	43	22	2	10
	5	23	19	21	13	2	5	28	18	3	12
	6	42	13	54	22	3	10	44	17	3	7
	10	24	9	50	13	2	7	55	22	3	12
	12	5	5	8	6	3	9	33	18	0	11
	48	32	8	20	8	3	9	41	13	1	7
	60	43	10	58	12	1	6	57	18	2	9
Total.....		264	102	346	120	30	78	374	153	16	84
Average.....		29.3	11.3	38.4	13.3	3.3	8.4	41.6	17	1.8	9.3
Average total gas.....		40.6		51.7		11.7		58.6		11.1	
CO ₂ /H.....		2.59		2.9		0.39		2.56		0.19	
Ghon-Sachs bacillus.....		8	16	3	7	0	0	6	18	0	8
Total gas.....		24		10		0		24		8	
CO ₂ /H.....		0.5		0.42		0		0.33		0	
B. oedematis-maligni.....	421	21	10	10	5	4	9	13	52	4	11
	485	14	10	11	9	2	10	33	17	3	10
	558	12	8	9	5	3	12	51	18	2	11
	560	5	3	15	5	4	10	14	5	10	13
Total.....		52	31	45	24	14	41	111	92	19	50
Average.....		13	7.8	11.3	6	3.3	10.3	27.8	23	4.8	12.5
Average total gas.....		20.8		17.3		13.6		50.8		17.3	
CO ₂ /H.....		1.68		1.88		0.32		1.2		0.38	
B. oedematis-maligni II.....		12	4	9	7	1	3	21	6	1	4
Total gas.....		16		16		4		27		5	
CO ₂ /H.....		3.0		1.3		0.33		3.5		0.25	
B. botulinus.....		9	5	10	5	0	6	11	8	2	8
Total gas.....		14		15		6		19		10	
CO ₂ /H.....		1.8		2.0		0		1.38		0.25	
B. coli.....		30	33	30	42	28	40	41	32	48	42
Total gas.....		63		72		68		73		90	
CO ₂ /H.....		0.97		0.71		0.70		1.28		1.14	

the broth, as is shown in the tables. This suggests the influence that training of the stock cultures may have on the gas-producing characteristic.

Our results thus far show that the minor sugars, etc., are of little value in the differentiation of the strains on the basis of total production of gas. Strains of the same organism obtained from various sources differ from one another considerably in their ability to attack the several carbohydrates and form gas from them. Again, some strains produce a certain amount of carbon dioxide while others produce none. Both these factors vary somewhat from experiment to experiment in the carbohydrate media with which only small amounts of gas are produced.

A study of the coefficients obtained with the carbohydrates from which the larger amounts of gas are formed — that is, dextrose, levu-

TABLE 4.—*Continued*
SUMMARIZED READINGS TAKEN FROM TABLE 3

Glycerin CO ₂ H	Sorbite CO ₂ H	Salicin CO ₂ H	General Average			
			Av. Total	CO ₂	H	CO ₂ /H
0 10	35 21	3 10	30.7	17.8	12.9	1.38
0 8	4 11	2 10	29.0	17.9	11.1	1.60
0 11	4 14	4 18	30.9	16.6	14.3	1.17
0 13	4 10	0 13	22.2	10.1	12.1	0.84
0 9	13 20	0 5	32.8	19.9	12.9	1.54
0 11	14 21	19 23	34.4	18.4	16.0	1.15
0 9	9 22	4 23	20.7	7.8	12.9	0.60
0 8	47 21	5 20	30.4	18.6	11.8	1.57
0 10	8 18	0 5	32.1	21.1	11.0	1.92
0 89	138 153	37 127				
0 9.9	15.4 17.6	4.1 14.1	29.4	16.7	12.7	1.32
9.9	33	18.2				
0.0	0.9	0.28				
0 +	0 9	0 0	8.6	2.1	6.5	0.33
+	0	0				
0	0	0				
0 8	6 18	0 19	23.8	7.3	16.5	0.44
11 16	32 28	3 23	29.6	13.6	16.0	0.85
0 9	8 18	8 25	24.9	11.6	13.3	0.88
11 26	8 12	0 8	19.3	8.4	10.9	0.77
22 59	54 76	11 80				
5.5 14.8	13.5 19	2.8 20	24.4	10.2	14.2	0.72
20.3	32.5	22.8				
0.37	0.71	0.14				
16 27	5 10	0 10	17.0	8.1	8.9	0.92
43	15	10				
0.60	0.50	0				
10 18	11 12	0 8	15.6	6.6	9.0	0.76
23	23	8				
0.55	0.92	0				
0 0	20 57	15 35	61.6	26.5	35.1	0.76
0	77	50				
0	0.35	0.42				

lose, mannose, maltose, saccharose, glycerin, sorbite, and salicin—shows several interesting results. In general it may be said that the malignant-edema strains produce less total gas, with a smaller proportion of carbon dioxide, than is produced by the symptomatic-anthrax strains. The Ghon-Sachs bacillus produces only a small amount of carbon dioxide—with most sugars, none at all.

With dextrose, levulose, and maltose, symptomatic-anthrax strains produce much the greater amount of gas, a major part of which is carbon dioxide.

With glycerin, symptomatic-anthrax strains produce no carbon dioxide at all, while malignant edema strains do. The latter also produce twice as much gas in this medium.

Malignant-edema-II strains, tho producing a smaller amount of gas, produce a greater proportion of carbon dioxide than Malignant-edema-I strains.

The gas shrinkage, especially in the gas of *B. coli*, as suggested by Keyes,⁵ would seem to be largely due to the diffusion of the more soluble carbon dioxid through the liquid to the open arm where the concentration is nearly zero. He finds that cultures of *B. coli* in a vacuum apparatus show a large amount of gas and a larger proportion of carbon dioxid than do cultures of the same organism in ordinary fermentation tubes. Since the action of *B. coli* is over in a comparatively short time and the collected gas stands for several days, there must be considerable loss by diffusion.

None of the 9 strains of the bacillus of symptomatic anthrax produced more hydrogen than carbon dioxid with dextrose. With this sugar, an average coefficient CO_2/H of 2.59 was obtained, a result contradicting the statement of Franklin and Haslam,⁶ that blackleg always produces more hydrogen than carbon dioxid with this sugar. It is not stated in their communication at what stage in the fermentation the gas was analyzed; a premature reading, such as one 72 hours after inoculation, would give a different carbon-dioxid factor from that obtained at the end of gas-production. Also the arbitrary use of a 4% sugar medium may have had some influence on the factors obtained.

Emphasis has frequently been placed on the characteristic odor of

TABLE 5
RESULTS OF TESTS FOR SUBSTANCES GIVING RISE TO CHARACTERISTIC PUTRID ODORS IN CULTURES

Strain No.	Hydrogen Sulfid	Methyl Mercaptan	Indol
<i>B. anthracis-symptomatici</i>	2	+	+++
	3	+	++
	4	+	++
	5	+	++
	6	—	++
	10	—	+
	12	+	+
	48	+	+
<i>Ghon-Sachs bacillus</i>	60	+	+
	1	—	±
	421	+	±
<i>B. oedematis-maligni</i>	485	+	++
	558	+	++
	560	+	++
<i>B. oedematis-maligni, II</i>	+	+	+
<i>B. botulinus</i>	+	+	+
<i>B. coli</i>	—	±	+++
<i>B. proteus</i>	+	+	+
Blank control.....	—	—	—

Tests used; for hydrogen sulfid, the lead acetate test; for methyl mercaptan, the isatin test⁷; for indol, Salkowski's reaction.

⁵ Jour. Med. Research, 1909, 16, p. 73.

⁶ Franklin and Haslam, Jour. Infect. Dis., 1916, 19, p. 462.

⁷ Bauer, Ztschr. f. physiol. Chem., 1902, 35, p. 346.

symptomatic-anthrax cultures as a means of differentiating them from those of malignant edema.^{8, 4} The general opinion is that cultures of symptomatic anthrax are of an aromatic acid odor, particularly like that of butyric acid, while those of malignant edema are of a vile putrid odor. The results of the olfactory test on our cultures agree with this general opinion when applied to young cultures, but with old cultures the reverse obtains. However, tests made for the substances credited with the production of the vile odor — indol, hydrogen sulfid, and methyl mercaptan — show that strain members of both the symptomatic-anthrax and malignant-edema groups originate these substances.

SUMMARY

B. anthracis-sympomatici produces more gas from dextrose, levulose, and maltose than *B. oedematis-maligni*, and a greater proportion of this is carbon dioxid. This is illustrated in the following summarized table (averages for 9 strains of *B. anthracis-sympomatici*, and for 4 strains of *B. oedematis-maligni*).

	Dextrose	Levulose	Maltose	Average
<i>B. anthracis-sympomatici</i>				
CO ₂	29.30	38.4	41.60	36.40
H.....	11.30	13.3	17.00	13.90
Total gas.....	40.60	51.7	58.60	50.30
CO ₂ /H.....	2.59	2.9	2.56	2.65
<i>B. oedematis-maligni</i>				
CO ₂	13.00	11.30	27.8	17.30
H.....	7.80	6.00	23.0	12.30
Total gas.....	20.80	17.30	50.8	29.60
CO ₂ /H.....	1.68	1.88	1.2	1.41

Training of seed cultures influences the gas-production of these organisms considerably.

Substances credited with the putrid odor are produced in cultures by strain members of both groups.

The gas-production of most of the strains occurs late and progressively up to about the 10th day. In some media, gas-production continues to a slight degree for some time after this.

An explosive mixture of gases, apparently containing hydrogen and no carbon dioxid, may be produced from "carbohydrate-free" broth.

⁸ von Hübner, Handb. d. pathogen. Mikroorganismen, 1912, 4, p. 808. Hiss and Zinsser, Bacteriology, p. 470.

TRANSMISSION OF PNEUMONIC AND SEPTICEMIC PLAGUE AMONG MARMOTS *

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The question of plague transmission among marmots was brought up at the time of the historic epidemic of pneumonic plague which raged in Manchuria during the winter of 1910-1911. The tarbagan, known as *Arctomys bobac*, was suspected of having played a part in the transmission of the disease, yet no experimental evidence was brought forth to show that this animal was in any way associated with plague. It was not until Strong¹ showed that tarbagans can be infected that we obtained the first important facts concerning the possible importance of these animals in the epidemiology of a dread disease. He demonstrated, in a general way, that they can take pneumonic plague if the organisms are sprayed in droplet form.

The experiments reported here were designed to elucidate this point and to determine the rôle played by the marmot in the spread of infection through contact and feeding on plague corpses.

The animals used were of a species closely related to the tarbagan, known as *Spermophilus citillus*. These ferret-like animals are very numerous in and about the city of Mukden, especially in the summer season. They frequent the grave yards, burrowing under the ground, not unlike the ground squirrel or American gopher. In size they approximate the rat. They are vicious, making good use of their long and exceedingly sharp teeth and claws. They can be easily trapped by pouring water into the burrows and catching them as they rush out to escape suffocation. With careful handling, they may live in captivity for a long time.

INHALATION EXPERIMENTS

The method of inoculation was made to conform as far as possible with the natural mode of infection with pneumonic plague.

A 24-hour-old agar slant culture of a moderately virulent strain of *B. pestis* was suspended in 10 c.c. of salt solution and sprayed from a graduated cylinder fitted with a very fine nozzle. Great care was taken to direct the

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* The experiments here summarized were conducted at Mukden Medical College, to the staff of which we are greatly indebted for their kindness and courtesy in giving us facilities for our work.

¹ Philippine Jour. Sc., 1912, 7, p. 225. Report of the International Plague Conference, Mukden, 1911.

spray in a fine cloud towards, but not into, the nasal passage. Altho a definite amount of culture was used in the two series of experiments, it was not possible to determine more than roughly, if at all, how much of the culture found its way into the respiratory tract. In the first series, 5 minims were sprayed and in the second, 10. A great portion of the spray obviously failed to inoculate, both because of the mechanical difficulties entailed in the technic and because of the position of the animals on the stage. It was necessary to clamp the neck in order to hold them down properly, so that in many instances normal breathing was out of the question. The errors due to the loss of most of the culture spray tend to make these results all the more striking. Before the spraying, the animals were covered with a wide piece of absorbent cotton soaked in cresol, to prevent droplets lodging on the fur, and after inoculation the head of each marmot was carefully wiped to remove extraneous organisms. In order to minimize the danger from droplet infection, a specially constructed box was used while the animal was being inoculated. This box enabled the operator to place the animal and stage in a compartment which had a glass top and which permitted of inoculation through an opening at the front end of the box. This aperture was at the level of the animal's head as it lay fastened on the stage and the spray, directed through the opening, reached the nose readily.

SERIES 1

In Series 1 the animals were placed in fleaproof cages, 9 by 15 by 20 inches, separated from the normal animals in some instances by a single partition, and in others by a complete four-walled screened compartment which stood in the center. The latter type of cage was used so that the normal animals might run about the central compartment and thus come in freer contact. Five minims were sprayed.

Experiment 1.—Two marmots were inoculated and 2 contacts placed in the adjoining compartment on the same day.

Both the treated animals failed to take plague. They were killed on the 20th day. No changes were noted in the organs, and smears from these were entirely negative. Of the two contacts, one died after 12 days. There were signs of inflammation in the lungs with congestion, and enlargement of spleen and liver; smears from these organs were negative. The second contact was killed after 21 days. No changes were observed in the organs, and smears from the organs and a blood culture were negative.

Experiment 2.—Two marmots were inoculated and screened from 4 normal animals placed in contact on the same day.

Contact 1 died after 7 days. Lungs inflamed, liver enlarged, spleen friable and necrosed, trachea and bronchi inflamed. Smears from heart, lungs, liver, and spleen showed great numbers of plague bacilli. A blood culture yielded *B. pestis*, and when injected in small amount into a normal marmot, it killed the animal in 48 hours with typical septicemic plague. Contact 2 died after 12 days. Organs congested, lungs severely involved, and the pleural cavity markedly inflamed. Bronchial inflammation marked. Smears from organs and blood positive for *B. pestis*. Contacts 3 and 4 died after 2 days with no morbid signs whatever.

Inoculated Marmot 1 died after 12 days with typical pneumonic and septicemic plague. Smears from organs and blood yielded great numbers of bacilli. Marmot 2 was killed after 18 days. No changes noted in the organs. Smears entirely negative.

In this experiment it is interesting that a contact succumbed 5 days before any of the inoculated animals died, and that of the latter two, one lived for 12 days while its mate, confined in the same cage, failed to take plague. It is very evident that here, as is the case among men, a difference in resistance must prevail. Apart from this we must take into account the different factors which tend to modify the chances of infection even by close contact.

Experiment 3.—Two marmots were inoculated and placed in the central compartment. Two normal contacts were placed in the cage after one day. The contacts were both alive and well on the 22nd day.

Inoculated Marmot 1 died after 10 days with typical pneumonic plague. Congestion of the organs and viscera, and enlargement of liver and spleen. Smears from blood and organs showed *B. pestis*. Marmot 2 was alive and well after 22 days.

Here we have another instance of chronic plague in which the animal lived beyond the ordinary 3-6 days after infection.

Experiment 4.—Three marmots were inoculated and placed in a central compartment as in the preceding experiment. Two normal animals were put into the surrounding space after 2 days and were then removed after being in contact for 5 days.

Contact 1 died after 15 days. Lungs showed slight inflammation and congestion; liver and spleen unchanged. Smears from organs and a blood culture negative. Contact 2 was alive and well after 22 days.

Inoculated Marmot 1 died after 5 days. Typical pneumonic plague. Liver greatly enlarged, spleen congested and friable, kidneys inflamed, cervical glands enlarged. Smears from blood and organs showed enormous numbers of plague bacilli. Marmot 2 died after 7 days. Lungs completely inflamed and congested; liver enlarged, friable, congested; spleen friable, hemorrhagic; kidneys pale, with petechiae. Smears from heart were negative, as were those from the spleen. The lungs showed great numbers of bacilli and a few were noted in the spleen tissue. Marmot 3, like the first, died of pneumonic and septicemic plague after 7 days.

Experiment 5.—Three marmots were inoculated and placed in a central compartment. After 3 days 2 normal animals were put into the surrounding space.

Contact 1 died after 3 days. Lungs inflamed; no changes in other organs. Smears from blood and organs and blood cultures negative. Contact 2 died after 7 days. Inflammation of lungs, enlargement and congestion of organs. Trachea and bronchi somewhat inflamed. Smears from organs and blood contained numerous plague bacilli.

Inoculated Marmot 1 died after 5 days. Lungs congested, acute double pneumonia. Liver and spleen enlarged, congested, and friable. Kidneys slightly inflamed and congested. Trachea and bronchi involved. Smears from blood and organs showed *B. pestis*, the greatest number being present in the spleen. Marmot 2 died after 7 days. Typical pneumonic and septicemic plague. Visceral congestion marked and the blood vessels engorged. Smears from the organs and blood showed enormous numbers of bacilli, the spleen being a solid mass of *B. pestis*. Marmot 3 died after 7 days. Postmortem findings were the same as in 2.

This first series of experiments indicates that pneumonic plague can be transmitted to the marmot rather readily, and that these animals, in turn, are capable of transmitting the disease to others. In one of

these experiments (5), it should be noted that one animal was able to infect a normal animal at least as early as 2 days after inhaling plague bacilli. From the results obtained in this set of experiments it is evident that the chances for infection by contact were minimized by the method of housing the animals, and the positive cases which resulted are therefore all the more surprising. Of 12 animals inoculated by inhalations, 8 died of typical plague pneumonia in from 3 to 7 days on an average. Of the contacts, 3 died and 7 survived. The percentage in the first instance is 66.6 and in the second, 30. Fifty percent of the animals which were exposed either directly or indirectly to the disease, died.

In order to insure more natural living conditions, such as normally prevail among these animals, a second series of experiments was conducted with the contacts placed in unscreened cages. Careful examination had already revealed the fact that fleas were very scarce on the marmots, and whatever insect transmission might occur, would be easily recognized from the resultant type of plague. This phase of the problem is now being studied and will make the subject of a separate report.

SERIES 2

Experiment 1.—Two marmots were inoculated. Three contacts were placed in the same cage after 24 hours.

Contact 1 died after 5 days. Lungs were inflamed throughout and contained fibrinous exudate. Liver and spleen markedly enlarged, congested, and stippled. Kidneys enlarged and inflamed. Trachea and bronchi inflamed. Smears showed numerous *B. pestis* in lungs, liver, and blood, and enormous numbers in the spleen. Contact 2 died after 12 days. Postmortem findings same as in 1, with additional pronounced visceral congestion. Smears from the organs showed numerous bacilli, with the greatest numbers in the lungs. The heart blood was negative in smear preparation, but yielded a pure culture on agar. Contact 3 died after 12 days. Postmortem findings were similar to those described. The greatest number of plague bacilli were present in the lungs.

Inoculated Marmot 1 died after 3 days. Typical plague pneumonia. Congestion and enlargement of spleen and liver. Smears from organs showed bipolar organisms and a few large rod-shaped bacteria. A culture from the heart blood was injected subcutaneously into a normal marmot and death ensued within 2 days from acute septicemic plague. Marmot 2 died after 9 days. Typical plague pneumonia and septicemia. Organs enlarged and congested. Visceral congestion marked. Plague bacilli present in enormous numbers in all the organs.

Experiment 2.—Two marmots were inoculated. Two contacts were placed in the same cage after 2 days.

Contact 1 died after 4 days. Lungs inflamed throughout and congested. Spleen and liver enlarged and congested. Visceral congestion pronounced.

Trachea and bronchi inflamed. Smears from organs and spleen showed numerous plague bacilli. Contact 2 died after 6 days. Postmortem findings the same as in 1. Smears from blood and liver negative. Numerous *B. pestis* in lungs and spleen.

Inoculated Marmot 1 died after 5 days. Lungs involved in upper lobes. Liver enlarged and necrotic. Spleen slightly enlarged, but not changed in appearance. Trachea and bronchi inflamed. Smears from lung showed enormous numbers of plague bacilli. Liver and spleen were a solid mass of organisms. Marmot 2 killed after 17 days. No changes noted in the organs. Smears from blood and organs entirely negative.

In this experiment, as in Experiment 2 of the first series, we have another striking example of individual difference in susceptibility. Three animals died of acute plague in from 3 to 6 days respectively, yet the fourth marmot, which was exposed to droplet infection and also kept in close contact for the entire period, survived. Moreover, a contact animal died a day sooner than the first of the inoculated marmots. It is important to bear in mind, however, that the methods of infection are different in each instance. A contact animal would in all likelihood receive a greater number of plague bacilli from the infective animal. Assuming that the dose thus received is equal to, or even less than that which was given to the inoculated animal, we must take into consideration the enhanced virulence of the culture as a result of initial passage through the host.

Experiment 3.—Two marmots were inoculated. Two contacts were placed in the same cage after 3 days.

Contact 1 died after 5 days. Lungs inflamed and greatly congested. Spleen and liver enlarged and congested. Inflammation of the bronchi and trachea marked. Smears from organs and blood showed enormous numbers of *B. pestis*. Contact 2 died after 6 days. Lungs inflamed and congested. Trachea and bronchi inflamed. Pronounced visceral congestion and engorgement of vessels. Smears from blood and organs yielded enormous numbers of bacilli, particularly in the spleen and lungs.

Inoculated Marmot 1 died after 5 days. Lungs slightly inflamed with involvement of upper lobes. Liver enlarged and slightly necrosed. Spleen somewhat enlarged. Bronchi involved. Smears from blood and organs showed enormous numbers of plague bacilli. Marmot 2 died after 6 days. Typical plague pneumonia. Marked visceral congestion. Greatly enlarged spleen. Congested liver. Smears from organs and blood contained numerous plague bacilli.

Experiment 4.—One marmot was inoculated. Two contacts were placed in same cage after 4 days.

Contacts 1 and 2 alive and well after 16 days.

Inoculated Marmot 1 died after 15 days. No marked changes in lungs or liver. Spleen slightly necrosed. Smears from heart blood, lungs, liver, and spleen negative. Culture from heart negative.

A summary of the results obtained in this series of experiments shows in conclusive manner that plague-infected marmots can readily transmit plague through the breath as is the case among men. Conditions which favor the propagation of the disease among the latter are in no way different for these animals. Close contact and moist surroundings seem to favor rapid spread from animal to animal. Of

7 marmots inoculated by inhalation, 5 died after from 4 to 6 days with acute pneumonic plague and septicemia — a percentage of 71. Nine contacts placed with infected marmots after periods varying from 1 to 4 days, showed a mortality of 77%. These 7 marmots died, on an average, after from 4 to 6 days' contact. Here, also, as in the preceding series, there was noted a remarkably short incubation period, with marked early infectivity on the part of the inoculated animals.

The postmortem findings indicate very clearly that pneumonic plague in marmots is not unlike that in men. The bacilli, entering the respiratory tract, lodge in the lungs and from this primary focus go over into the circulation to cause a generalized septicemia. Most striking are the gross pathologic changes in the organs, chiefly the lungs. Extreme congestion and inflammation with fibrinous exudate characterize the latter, and associated with this is pronounced inflammation of the pleural cavity. Enlargement of the spleen and liver is frequent, tho not constant, and visceral congestion is prominent. No instances of axillary or inguinal gland-involvement were observed, altho in a few cases the cervical glands were enlarged and upon microscopic examination of smears, showed plague bacilli in goodly numbers. Inflammation of the trachea and bronchi occurs with marked regularity.

Bacteriologic examination demonstrated that altho the lungs may contain enormous numbers of bacilli, yet this organ is not exclusively selective. In a fair proportion of cases in which the lung had few organisms, the spleen invariably teemed with them. This was also noted when blood smears were not particularly full of *B. pestis*. The number of organisms present in any one organ at a given time seems to depend on a variety of circumstances, not the least of which appears to be the resistance of the animal in question. Some animals, it was noted, may evidence a distinct toxemia without marked bacteriemia. In analogous fashion, a few of the marmots may offer such low resistance to the disease that they succumb to it before any very marked changes appear in the organs.

Of great interest is the fact that plague may exist in chronic form among marmots. That they can live for 9 or 12 days with pronounced plague and be capable of conveying infection to other animals, is a fact of the utmost importance from an epidemiologic standpoint.

SUSCEPTIBILITY TO INFECTION

Plague septicemia, as seen, results readily from plague pneumonia. In order that we might study the susceptibility of marmots to this type

of plague, a number of animals were inoculated subcutaneously with varying doses of bacilli. All the animals died of acute septicemic plague with slight, if any, signs of bubonic infection. That these animals are very susceptible to plague septicemia follows from this experiment. The culture used was only moderately virulent and had been growing on agar for several generations. A 72-hour growth on small agar slants was used for inoculation. Animals numbered 6 to 10, inclusive, were inoculated with the same strain after it had passed once through a marmot.

Marmot	Dose (slant)	Result	Postmortem Findings
1.....	1/20	Death, 2 days	In all intense congestion at site of inoculation. Spleen and liver enlarged and congested. Smears from blood and organs showed enormous numbers of plague bacilli.
2.....	1/40	Death, 2 days	
3.....	1/80	Death, 6 days	
4.....	1/40	Death, 4 days	
5.....	1/80	Death, 5 days	
6.....	1/80	Death, 36 hours	
7.....	1/80	Death, 36 hours	
8.....	1/80	Death, 48 hours	
9.....	1/80	Death, 48 hours	
10.....	1/80	Death, 48 hours	

PLAGUE TRANSMISSION BY FEEDING

Experiment 1.—Three marmots were fed with liver and spleen taken from a guinea-pig which had died of plague after 56 hours.

Marmot 1 died after 3 days. Liver and spleen congested. Stomach inflamed. Smears from lungs, liver, spleen, and blood showed *B. pestis*. A blood culture, injected into a normal marmot, killed the animal within 36 hours, the autopsy showing acute plague with *B. pestis* in all the organs and blood. Marmot 2 died after 3 days. This animal was eaten by the third marmot before a postmortem examination could be made. Marmot 3 died after 4 days. Slight visceral congestion. Inflammation of the gastric mucosa. Smears from blood and organs showed *B. pestis*. Stomach scrapings were full of plague bacilli.

Experiment 2.—Three marmots fed with lung, liver, and spleen taken from a marmot which had died of acute plague.

Marmot 4 died after 2 days. Congestion of liver and spleen. Marked visceral congestion and intense inflammation and congestion of the gastric mucosa. Smears from organs and stomach lining showed great numbers of plague bacilli. Marmot 5 died after 4 days. Liver and spleen congested and enlarged. Visceral congestion marked. Gastric mucosa intensely inflamed. Smears from blood and organs gave enormous numbers of *B. pestis*. Marmot 6 killed after 14 days. Postmortem examination showed no changes in any of the organs or glands. A very slight area of old inflammation was noted in the gastric mucosa. No organisms could be demonstrated in smears from blood or organs. This animal had been fed twice with plague material with an interval of 1 week between feedings.

This series, tho small, demonstrates that marmots may transmit plague by feeding on plague carcasses. The animals are carnivorous

and eat their own mates as soon as death supervenes. Death, by feeding, takes place within 4 days, and is apparently not hastened by greater amounts of ingested plague-infected material. The most striking change in the animal body is noted in the stomach, where intense inflammation of the gastric mucosa occurs. The spleen and liver show the usual changes attendant on plague.

That individual differences in susceptibility may exist, is well exemplified in the case of Marmot 6, which failed to take plague, tho it had been given a large amount of highly infected material.

MICROSCOPIC EXAMINATION

The experiments on the small marmot (*Spermophilus citellus*) mentioned, particularly with regard to pulmonary plague infection by contact, are perhaps the first that have been recorded. The feeding experiments are especially interesting because many workers, including Strong, have denied the possibility of plague transmission by that means. The histologic changes observed in the lesions of human pulmonary plague have been fully described by various writers, including Albrecht and Ghon² (1898-1900), Childe³ (1898), Strong¹ (1912), Fujinami⁴ (1912), and Wu Lien Teh and Woodhead⁵ (1913). We preserved and examined a considerable number of specimens from the animals used in these experiments, but, as the microscopic changes in the inhalation experiments differ in no material way from those already described in the case of human pulmonary plague, we shall refer to them only briefly.

Lung.—In acute pulmonary plague (i. e. when the animals died in from 2 to 5 days after infection), sections of the lung showed intense congestion of the blood vessels. The part of the lung tissue adjacent to the pleura was marked by much leukocytosis and even hemorrhage, and areas of collapse could be seen. The small bronchi were filled with mucoid substance, and some were practically choked with pure growths of plague bacilli. Around the inflamed bronchi and bronchioles were patches of pneumonia, harboring numbers of bacilli in the capillaries and alveoli. No fibrinous lymph coagulum was noted.

In specimens obtained from 2 cases which had died 12 days after contact with infected animals, the lung tissue showed somewhat different changes. Here hemorrhage and congestion were not so marked, and bronchopneumonic patches were scanty. The alveoli displayed extensive signs of collapse, and the bronchi signs of inflammation and thickening. Plague bacilli were not nearly so numerous as in acute plague.

Liver.—In the acute form, sections of the liver showed a picture of acute red atrophy, the central lobular vein being much distended, and the portal capillaries swollen. The hepatic cells were markedly "cloudy" and granular, but vacuolation, except in a few areas, had not set in earnestly. Hemorrhages were noted everywhere.

In specimens obtained from the chronic cases, the liver substance showed

² Centralbl. f. Bakteriöl., 1899, 26, p. 362.

³ Brit. Med. Jour., 1897, 1, p. 1215; 1898 2, p. 858. Report of Indian Plague Commission, London, 1900.

⁴ Report of the International Plague Conference, Mukden, 1911.

⁵ Jour. Path. and Bacteriol., 1913, 19, p. 1.

very characteristic signs of degeneration. The central lobular vein was not so distended, and hemorrhage was not so marked. A large portion of the hepatic cells appeared to have lost their contents, so advanced was the vacuolation and loss of nuclear substance. In fact, the whole section stained badly with hematin. Plague bacilli were seen with difficulty.

Spleen.—Here also the changes observed in the acute and chronic disease were characterized by much more congestion in the former than in the latter. The number of bacilli encountered was also greater in the acute form, and the Malpighian bodies were larger and stood out more distinctly.

Kidney.—As in the case of the liver, the kidney showed far more extensive signs of degeneration in the chronic, than in the acute cases. There was very little thickening of the capsule in either case, but the glomeruli were swollen considerably. In the chronic cases the cells of the tubules had lost the greater part of their substance, and in several places only the basement membrane was seen, so great had been the disintegration. More hemorrhage was noted in the kidney than in other organs in the chronic cases.

Heart.—The muscular tissue showed edematous changes, the striations being more indistinct than usual, and the muscle fibers broken in places.

Lymphatic Glands.—Both cervical and inguinal glands were examined, but no changes were shown other than those hitherto described in ordinary plague. Organisms were present in lesser numbers than in bubonic plague, and, in the chronic form, were sometimes not seen at all.

Stomach.—So many observers have denied the existence of infection by the alimentary canal that a little more attention may be devoted to the changes observed in this organ. As stated in the preceding section, of 6 marmots fed on plague-infected viscera 5 died—4 definitely of plague, the 5th being eaten by its fellows before an examination could be made. The one animal which survived, after 14 days was killed; it showed no signs of infection. Post-mortem examination in all cases was made within a few hours after death. The stomach in all the infected animals showed definite signs of acute inflammation, which was most marked at the pyloric end and commencement of the duodenum. Red patches, denoting hemorrhage, and small areas of disintegration were clearly seen. Pieces of the stomach at the pyloric end were removed from Cases 4 and 5, and prepared for microscopic examination. Formalin was used as fixing agent, and the paraffin sections were stained with both alum hematin plus eosin, and dilute Giemsa, as follows: Stain in dilute Giemsa (1 part Giemsa Grübler solution in 10 parts distilled water) for 6 hours. Decolorize in weak acetic acid (5 drops in 100 c.c. distilled water). Wash in distilled water. Blot and clear in xylol. Plague bacilli, when present, are stained deep-blue in the tissues by this method.

The gastric mucosa showed marked changes under the microscope. The mucous glands were intensely inflamed, and hemorrhages could be seen both inside and around them. Clots with fibrin were also encountered, sometimes firmly adherent to the underlying glands. At places large areas of glandular tissue had given way, revealing open ulcers with much leukocytic infiltration and ruptured blood vessels around the edges. Apparently the large oxyntic cells were first cast out, for here and there numbers of them were found on the surface intermixed with leukocytes. In other parts, where disintegration had been extensive, only granular debris was left. The cells of the glands were swollen and granular, and where inflammatory changes were most marked they appeared broken up. Plague bacilli were met with in varying numbers amidst the glands, and were most evident on the surface of the necrotic areas. The

submucous coat was thickened, the blood vessels supplying the glands being much distended and filled with corpuscles. The inner circular muscular coat was also congested, and large clumps of plague bacilli were seen distributed among the fibers, especially in the neighborhood of blood vessels. The fibers themselves appeared swollen, but no signs of disintegration could be made out. The outer longitudinal muscular coat seemed also swollen, but very few bacilli were met with in this region. The peritoneal coat was slightly infiltrated in certain parts.

In the sections obtained from Marmot 5, the surface of the mucous coat seemed to be largely covered with an organized coagulum of mucoid tissue of varying thickness. Where the clot had broken off, the mucous glands showed necrotic changes similar to those described, and the surrounding blood vessels were greatly distended. Plague bacilli were present in large numbers both inside and outside the clot, and in the granular debris of the mucous glands.

SUMMARY AND CONCLUSIONS

FIRST SERIES		SECOND SERIES	
Animals inoculated..	12	Deaths..	8
Contacts	10	Deaths..	3
Total	22	Total	11
		Animals exposed to plague.....	38
		Deaths	23

Of the marmots placed in contact with marmots which had received inoculation by inhalation, 52.6% developed pulmonary plague and died on an average within from 4 to 6 days.

Early infectivity on the part of the inoculated animals and a short incubation period characterize the transmission.

Pulmonary plague can be transmitted readily to the small marmot (*Spermophilus citellus*), and these animals are capable, in turn, of transmitting the same disease through the respiratory passages.

Septicemic plague can be developed in marmots very easily as a result of respiratory infection, and also by direct subcutaneous inoculation with small amounts of culture.

The marmot can take plague by way of the alimentary tract and can spread the disease by feeding on plague-infected carcasses. The microscopic lesions observed in these cases are characteristic.

PLAGUE POISONS AND VIRULENCE*

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The brief note here given represents a few experiments which were made incidental to a study aiming at immunity to septicemic and pneumonic plague.

Friedberger¹ discovered that powerful poisons could be produced if complement, as present in normal guinea-pig serum, was allowed to act on suspensions of bacteria. These poisons, or "anaphylatoxins," as he named them, give acute shock on injection and cause the death of the animal. Recently Zinsser² showed that animals may acquire distinct tolerance of such poisons and survive large doses of the "proteotoxins" without evidencing any noticeable shock after the preliminary dose, which is so measured as to give a slight shock to the animal. It was also demonstrated that these products apparently possess aggressin-like properties, which, if injected in combination with sublethal doses of bacilli, such as typhoid bacilli, cause death through a resulting bacteriemia.

In these experiments I have tried to obtain Zinsser's proteotoxins from plague bacilli in an attempt to immunize animals with this poison. Contrary to results obtained with other organisms, it was impossible to develop a poison which would give the slightest shock when injected intravenously. Curiously enough, all the animals so treated died of acute plague after several days. Postmortem examination revealed the presence of plague bacilli in great numbers in the blood and organs. Evidently the few bacilli which remained in the supernatant fluid after prolonged centrifugation of the serum plus the organisms, had become more virulent and an aggressive action, as noted by Zinsser, had occurred.

The culture used in these experiments was an avirulent Shanghai strain of *B. pestis* that had been growing on artificial media in this laboratory for 1½ years. The method of procedure was, in general, as follows: An 18- to 24-hour-old culture, grown on agar, was washed off with 1 c.c. of salt solution

* Received for publication November 7, 1916.

¹ Berl. klin. Wehnschr., 1910, 47, pp. 1490, 1922.

² Jour. Exper. Med., 1914, 20, p. 387.

and incubated with normal guinea-pig serum for various periods at 37 C. At the end of these periods the emulsions were centrifugated at high speed for 1 hour or more, and the supernatant fluids injected intravenously into guinea-pigs.

Experiment 1.—A 24-hour-old culture was incubated with guinea-pig serum (10 c.c.) for 3½ hours.

Guinea-Pig	Weight	Dose	Result
1	432 gm.	1.0 c.c.	No shock. Died after 7 days. Organs and blood full of plague bacilli.
2	411 gm.	0.5 c.c.	No shock. Lived.
3	423 gm.	2.0 c.c.	No shock. Died after 6 days. Same as 1.

In this experiment no shock was produced with doses varying from 0.5 to 2 c.c. Since the incubation period used here seemed rather short, Experiment 2 was made.

Experiment 2.—An 18-hour-old culture of *B. pestis* was washed off with 1 c.c. of salt solution and incubated with 6.5 c.c. of normal guinea-pig serum for 5½ hours.

Guinea-Pig	Weight	Dose	Result
1	310 gm.	2.0 c.c.	No shock. Died after 4 days with acute plague. All organs and blood contained enormous numbers of bacilli.
2	365 gm.	2.5 c.c.	No shock. Died after 5 days. Plague bacilli in all organs and blood.
3	295 gm.	1.5 c.c.	No shock. Died after 5 days. Acute plague as in 1 and 2.

A longer incubation period had no effect on the production of bacillary poison. In Experiment 3 the amount of culture was increased.

Experiment 3.—Two agar cultures, 24 hours old, were washed off with 0.5 c.c. of salt solution, respectively, and incubated with 6 c.c. of guinea-pig serum for 5½ hours.

Guinea-Pig	Weight	Dose	Result
1	198 gm.	1.5 c.c.	No shock. Died after 1 day. No plague bacilli found in smears or in cultures.
2	227 gm.	2.0 c.c.	No shock. Died after 3 days. Typical plague infection. All organs and blood contained great numbers of <i>B. pestis</i> .
3	276 gm.	2.5 c.c.	No shock. Died after 4 days. Same as 2.

An increased amount of culture had no effect on the action of complement so far as producing a visible shock was concerned. In this experiment, as in the preceding ones, some of the animals showed symptoms of discomfort and illness. If we remark the accelerated death in this series of guinea-pigs, it seems very likely that the poison was more marked. When we say "no shock," we wish to imply that the animal did not show the usual symptoms attending anaphylactic or anaphylatoxic poisoning, altho in a few instances very slight tokens of illness could be detected. In Experiment 4 a greatly prolonged period of incubation was tried.

Experiment 4.—A 24-hour-old culture was washed off with 1 c.c. of salt solution and incubated with 9 c.c. of guinea-pig serum for 16 hours at 37 C. The mixture was centrifugated at high speed for 2 hours and then injected in the same manner as before. A control was incubated with salt solution for the same period and likewise injected.

Guinea-Pig	Weight	Dose	Result
1	243 gm.	3.0 c.c.	No shock. Appeared sick. Died after 36 hours. The subcutaneous vessels engorged. Glands inflamed and enlarged. Spleen enlarged and slightly necrosed. Edema marked. No plague bacilli demonstrated in the blood or organs.
2	237 gm.	2.5 c.c.	No shock. Appeared sick. Died within 36 hours. Postmortem findings same as in 1.
3*	267 gm.	3.0 c.c.	No shock. Well. Died after 36 hours. Postmortem findings same as in 1 and 2.

* Control.

Here there is evidence of poisoning. The animals showed all signs of a toxemia and no plague bacilli were found in any of the organs or in the blood. It will be seen, however, that the poison produced did not give any actual shock and that the salt-solution control, moreover, acted exactly like the serum from the treated cultures. These results show, therefore, that in this instance a prolonged incubation period effected autolysis of the bacteria and liberated the endotoxins.

The marked absence of shock in the foregoing experiments did not warrant the supposition that a poison, if at all produced by the action of complement, could by itself exert such strong aggressive action as to kill animals which were injected with exceedingly minute amounts of plague bacilli in themselves not virulent. The few organisms which were not removed by centrifugation could not, by any stretch of the imagination, be held responsible for the death of the animals. In Experiment 5 there is decided evidence that contact with normal guinea-pig serum enhances the virulence of the plague bacilli.

Experiment 5.—A 24-hour-old culture of the avirulent strain used in the preceding experiments was washed off with 1 c.c. of salt solution and incubated with normal guinea-pig serum in the proportions of 0.2 c.c. of the bacterial suspension to each 2 c.c. of the serum. At the end of each incubation period the emulsions were centrifugated, the sediment carefully washed in order to remove all traces of serum, and the bacteria resuspended in salt solution. Injections were then given intraperitoneally with graded doses of the organisms.

Guinea-Pig	Weight	Dose	Incubation	Result
1	342 gm.	0.2 c.c.	5 hr.	Died within 30 hours of acute plague. All organs and blood contained enormous numbers of bacilli
2	305 gm.	0.2 c.c.	22 hr.	Died after 28 hours. Same findings as in 1
3	510 gm.	0.1 c.c.	5½ hr.	Died after 44 hours. Acute plague. Postmortem findings same as in 1
4	576 gm.	0.1 c.c.	5½ hr.	Died after 72 hours. Same as preceding
Controls				
1	344 gm.	0.1 c.c.	Lived. Well
2	250 gm.	0.1 c.c.	Lived. Well
3	348 gm.	0.2 c.c.	Lived. Well
4	372 gm.	0.2 c.c.	Lived. Well
5	350 gm.	0.3 c.c.	Died after 5 days
6	305 gm.	0.4 c.c.	Died after 3 days

In this experiment there is conclusive proof that contact with the serum has enhanced the virulence of the culture. If the control animals are compared with the heaviest test animal, it will be noted that the latter succumbed to at least one-seventh the dose required to kill the former in approximately the same time. This increase in virulence by itself, however, cannot account for the death of the animals treated with plague proteotoxin containing a few bacteria. It is evident that the aggressive action of the poison goes hand in hand with the increase of virulence to bring about the results noted.

These experiments repeated with a virulent strain of plague bacteria, gave identical results except that the animals died sooner after receiving the dose of proteotoxin. Naturally this was to be expected, because the culture was far more virulent and the few remaining bacilli in the guinea-pig serum, when further increased in virulence, brought about an overwhelming septicemia.

Whether or not the same results obtain when sensitized cultures are used, remains to be seen. This phase of the problem is being studied and will make the subject of a separate report.

The advantages possessed by such a poison for immunization purposes become very evident when we consider that we are likely to

obtain the active principle of the organism by resorting to such a method. From previous work done in immunity to bubonic plague, we have all reasons to believe that the more nearly we can approximate a virulent, or at least, a living culture for purposes of immunization, the more hopeful will be the results. Strong's method of inoculating with a living avirulent strain demonstrates this point but is open to the objection that a living culture is uncertain, if not dangerous, since one is ignorant as to the fate of such bacilli after they are injected into the human body. The experiments here reported confirm the likelihood of just such an unfavorable outcome. Assuming that contact with a normal serum, under body conditions, simulates, in a general way, what might occur in the human body, we should not hazard such a method very freely for large-scale immunization. Lustig and Galeotti,³ and more recently Rowland,⁴ have demonstrated the value of active bacillary substances for immunization in bubonic plague. These authors obtained excellent results with their so-called "nucleoproteins" of the plague organism.

The value of plague proteotoxins in pneumonic and septicemic plague is now being studied, and we hope to report on this work in a future communication.

³ Report of the International Plague Conference, Mukden, 1911.

⁴ Jour. Hyg., 1912, 12, p. 344.

THE EFFECT OF VARIOUS CHEMICAL SUBSTANCES ON THE HEMOLYTIC REACTION *

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Hektoen¹ in 1903 pointed out that some of the inorganic salts, such as CaCl_2 , MgCl_2 , BaCl_2 , NaSO_4 , $\text{Na}_2\text{C}_2\text{O}_4$, and $\text{Na}_2\text{C}_2\text{H}_3\text{O}_6$, inhibit the hemolytic reaction when present in certain concentrations. This work was extended by Manwaring² and by Hektoen and Ruediger³ to cover bacteriolysins, as well as hemolysins, and a much larger number of salts. They found that small doses of M/8 solutions of many salts prevent the lysis of red corpuscles and of bacteria by various sera, and concluded that the antilytic action is the result of the action of these salts on the complements. Arkin⁴ (1913), in reporting on the effect of several substances on opsonins, mentions that lactic acid in certain concentrations not only inhibits phagocytosis, but prevents complement from combining with amboceptor in the hemolytic reaction. I have noted in a previous report⁵ that a temporary drop in complement occurs during the Eck-fistula operation, which seems to be the result of the anesthesia. It is generally known that an acidosis usually results from the administration of ether or chloroform,⁶ especially following chloroform anesthesia, and perhaps this acidosis is a factor in causing the temporary drop in complement during Eck-fistula operations, inasmuch as the drop is much greater under chloroform than under ether anesthesia. Wendelstadt⁷ showed that in the presence of a small amount of amboceptor glycogen suspends hemolysis by acting on the complement. Hektoen and Ruediger mention the work of Camus and Pagniez,⁸ Neisser and Doebling,⁹ and others, showing the ability of uremic serum to inhibit hemolysis by normal serum. Walker¹⁰ and others have reported the presence of complement-fixing substances in the serum of a certain percentage of diabetics. Cumming¹¹ has shown that the sign of the Wassermann reaction may be changed by the influence of various salts, acids, and alkalies on the hemolytic system.

The work of this paper is an investigation of the effect of lactic acid, hydrochloric acid, sodium bicarbonate, and acetone on the complement content of rabbits *in vivo*; of ether and chloroform anesthesia

* Received for publication November 16, 1916.

¹ Trans. Chicago Path. Soc., 1903, 5, p. 303.

² Jour. Infect. Dis., 1904, 1, p. 112.

³ Ibid., p. 379.

⁴ Ibid., 1913, 13, p. 408.

⁵ Sherwood, Smith, and West, Jour. Infect. Dis., 1916, 19, p. 682.

⁶ Woolley, Jour. Lab. and Clin. Med., 1916, 1, p. 782.

⁷ Centralbl. f. Bakteriol., I. O., 1903, 34, p. 83.

⁸ Camus and Pagniez, Compt. rend. Soc. de biol., 1901, 53, p. 730.

⁹ Berl. klin. Wehnschr., 1901, 38, p. 593.

¹⁰ Jour. Am. Med. Assn., 1916, 66, p. 488.

¹¹ Jour. Infect. Dis., 1916, 18, p. 151.

on the hydrogen-ion concentration and complement content of the blood of normal rabbits; and lastly of carbon dioxid, lactic acid, hydrochloric acid, uric acid, urea, benzoic acid, tartaric acid, hydrazin sulfate, acetic acid, acetone, ether, and chloroform on the hemolytic reaction in vitro. It is hoped to extend this work to cover many other substances such as aceto-acetic acid, beta oxybutyric acid, etc., which are found under pathologic conditions, and also perhaps to cover some of the aminoacids. The group under discussion are also being tried out with reference to their effect on antibody-production and on hemagglutinins and bacterial agglutinins.

EFFECT OF ETHER AND CHLOROFORM ANESTHESIA ON RABBIT
COMPLEMENT IN VIVO

Three series of rabbits were selected and the C_H^+ and the complement titer determined for each rabbit. The animals in Series 1 were then anesthetized with ether for varying lengths of time and the C_H^+ and complement content of their sera again determined. The animals in Series 2 were saturated with sodium bicarbonate by intravenous, subcutaneous, and intraperitoneal injections of a 2% solution of sodium bicarbonate, and the C_H^+ and complement content of their sera determined. The animals were then put under ether anesthesia for varying lengths of time, and the C_H^+ and complement content again determined. Series 3 was given chloroform for varying lengths of time, and data obtained as before. The results were as follows:

The hydrogen-ion concentrations¹² were determined by the colorimetric method,¹³ the gas-chain method not being available. To 1 c.c. of boiled double-distilled water in a hemolytic test tube was added 1 drop of the indicator, the tubes were shaken, then 0.1 c.c. of the serum was added, and the tubes were again shaken, and read.

Since a hydrogen-ion concentration (C_H^+) of 10^{-7} is a truly neutral solution—that is the concentration of the hydroxyl ions (C_{OH}) is 10^{-7} —it will be noticed that rabbit blood seems to be slightly alkaline (10^{-8}) or neutral (10^{-7}) normally. Following ether anesthesia there is an increase in acidity. A drop in complement seemed to occur only when this increase in acidity passed the neutral point. In Series 2 the injection of sufficient sodium bicarbonate to change the C_H^+ from 10^{-7} to 10^{-10} did not cause any variation in the complement content. Neither

¹² Washburn, Proc. Illinois Water Supply Assn., 1910, p. 93.

¹³ Kelley, Jour. Lab. and Clin. Med., 1915, 1, p. 194. Wilson and Hutchinson, Jour. Biol. Chem., 1909, 4, 346.

TABLE 1
SERIES 1, ETHER ANESTHESIA

Rabbit	C_H^+	Complement Titer, Undi- luted Serum (c.c.)	Time of Anesthesia (min.)	C_H^+	Complement Titer, Undi- luted Serum (c.c.)
1	10^{-8}	0.05	10	10^{-6}	0.10
2	10^{-9}	0.03	15	10^{-7}	0.03
3	10^{-8}	0.03	20	10^{-6}	0.15
4	10^{-8}	0.08	45	10^{-5}	0.25

TABLE 2
SERIES 2, SODIUM BICARBONATE AND ETHER ANESTHESIA

Rabbit	C_H^+	Comple- ment Titer (c.c.)	Material Injected (Sodium Bi- carbonate)	C_H^+	Comple- ment Titer (c.c.)	Time of Anes- thesia (min.)	C_H^+	Comple- ment Titer (c.c.)
1	10^{-7}	0.05	20 c.c. 2% solution	10^{-10}	0.05	30	10^{-7}	0.05
2	10^{-7}	0.03	20 c.c.	10^{-9}	0.03	45	$10^{-6.5}$	0.08
3	10^{-7}	0.03	1/12 gr. morphin	10^{-8}	0.03	15	10^{-8}	0.05
4	10^{-7}	0.05	40 c.c.	10^{-10}	0.05	30	10^{-7}	0.05
5	10^{-6}	0.30	30 c.c.	10^{-8}	0.05	15	10^{-7}	0.05

TABLE 3
SERIES 3, CHLOROFORM

Rabbit	C_H^+	Complement Titer (c.c.)	Time of Anesthesia (min.)	C_H^+	Complement Titer (c.c.)
1	10^{-8}	0.03	10	10^{-7}	0.03
2	10^{-8}	0.03	30	10^{-5}	0.40
3	10^{-8}	0.05	15		Killed
4	10^{-7}	0.05			Killed

did the complement titer drop when those animals which had been saturated with sodium bicarbonate were given ether anesthesia for varying lengths of time. The hydrogen-ion concentration did not return much past the neutral point of 10^{-7} . It is suggestive that Rabbit 5 had very little complement and a C_H^+ of 10^{-6} ; after the animal had been saturated with sodium bicarbonate, the complement content returned to normal and the C_H^+ dropped to 10^{-8} . It has been stated that morphin prevents acidosis. Rabbit 3, Series 2, which received $\frac{1}{12}$ gr. of morphin and then ether anesthesia, showed only a slight drop in complement content; the C_H^+ remained on the alkaline side of 10^{-7} , being 10^{-8} . These experiments, while not conclusive, are suggestive.

EFFECT OF LACTIC ACID AND HYDROCHLORIC ACID ON
COMPLEMENT IN VIVO

For this purpose 4 rabbits were selected, 2 for treatment with lactic acid and 2 for treatment with hydrochloric acid. The complement content and C_{H^+} for each were determined and then 2 received each the equivalent of 0.5 c.c. of N/1 lactic acid intravenously, and 1 c.c. intraperitoneally, and the other 2 received each corresponding amounts and equivalents of N/1 hydrochloric acid. The C_{H^+} and complement content were then determined and greater amounts of material injected. The results were as given in Table 4.

TABLE 4
DATA ON RABBITS INJECTED WITH LACTIC ACID

Rabbit	C_{H^+}	Complement Titer (c.c.)	Amount of Lactic Acid Injected (c.c.)	C_{H^+}	Complement Titer (c.c.)
1	10^{-7}	0.10	1.5	10^{-7}	0.08
	10^{-7}	0.05	6.0	10^{-7}	0.05
	10^{-7}	0.05	4.0	10^{-7}	0.05
2	10^{-7}	0.08	1.5	10^{-7}	0.05
	10^{-7}	0.05	6.0	10^{-7}	0.05
	10^{-7}	0.05	4.0	10^{-7}	0.05

The injection of lactic acid did not cause a drop in complement; it rather favored an increase in the complement content. The fact that the body is able to oxidize many of the organic acids, including lactic acid, over to the carbonates and that they then act as alkalis, would account for the similarity of action between lactic acid and sodium bicarbonate.

Twenty-four hours after these data were obtained, both animals received additional doses of lactic acid; death resulted. In administering the acid the desired amount of normal solution was pipetted out and diluted 15 or 20 times with isotonic salt solution, and this diluted solution injected.

The results of the action of hydrochloric acid are given in Table 5.

TABLE 5
DATA ON RABBITS INJECTED WITH HCl

Rabbit	C_{H^+}	Com- plement Titer (c.c.)	Material and Amount Injected	C_{H^+}	Com- plement Titer (c.c.)	Remarks
1	10^{-8}	0.03	5 c.c. N/20 HCl	$10^{-0.5}$	0.10	Died
	$10^{-0.5}$	0.10	15 c.c. N/20 HCl	10^{-0}	0.15	
	10^{-0}	0.15	20 c.c. 2% Na_2HCO_3			
2	10^{-8}	0.05	5 c.c. N/20 HCl	10^{-7}	0.10	Died
	10^{-7}	0.10	15 c.c. N/20 HCl	10^{-0}	0.15	
	10^{-0}	0.15	20 c.c. 2% Na_2HCO_3			

The results are in keeping with the fact that the body is unable to oxidize the mineral acids. The drop in complement did not occur immediately, but several hours after the administration of the acid. Normal variation in the rabbit complement might also account for a part, at least, of the variation. The complement might be affected also by any CO_2 liberated as the result of injection of dilute HCl and its action on the carbonates present in the body. Accordingly, fresh complement was taken and CO_2 bubbled through it for varying lengths of time up to 15 minutes. The hemolytic reaction was inhibited as long as CO_2 was present in an appreciable amount, but proceeded as soon as the CO_2 was driven off.

Since in a typical acidosis, such as one finds in diabetes, aceto-acetic acid, beta oxybutyric acid, acetone, and CO_2 are increased in the blood stream, it was next decided to try out acetone, aceto-acetic acid and beta oxybutyric acids not being available. Two rabbits were each given an injection of a 2% aqueous solution of acetone every hour for several hours, 20 c.c. being given at each injection. The C_{H}^+ and complement content were determined before and after each injection and on the half hour between. The C_{H}^+ remained from the beginning at 10^{-8} for Rabbit 1 and at 10^{-7} for Rabbit 2, and the complement titer did not vary from the original normals; that is, 0.03 c.c. contained 1 unit in Rabbit 1 and 0.05 c.c., 1 unit in Rabbit 2. Quantitative determinations of the amount of acetone in the blood stream or urine were not made. Again, these results might be explained on the basis of a not sufficient concentration of acetone in the blood stream, as well as that acetone by itself has no effect on complement in vivo. The former is a plausible explanation in view of the work of Manwaring, Hektoen, and others on the antilytic action of various salts when the concentrations were found to be important in vitro.

EFFECT OF DIFFERENT CONCENTRATIONS OF THE REAGENTS ON THE HEMOLYTIC REACTION

It was next decided to determine the effect of different concentrations of lactic acid, hydrochloric acid, uric acid, benzoic acid, tartaric acid, urea, hydrazin sulfate, acetic acid, acetone, ether, and chloroform on the hemolytic reaction.

In doing this it was thought necessary to determine the effect, if any, of each of these reagents on red blood cells, complement, and amboceptor, respectively. In working with lactic and hydrochloric acids, normal and decinormal solutions were carefully made up and standardized, and, when necessary, dilu-

TABLE 6
EFFECT OF THE VARIOUS REAGENTS ON HUMAN BLOOD CELLS

Reagent	Amount (c.c.) Used and Results			
	1	2	3	4
N/25 lactic acid.....	0.5 —	0.4 —	0.3 —	0.2 —
N/30 HCl.....	0.5 —	0.4 —	0.3 —	0.2 —
Saturated solution of uric acid.....	0.8 ++++	0.7 ++++	0.6 ++++	0.5 ++++
M/1 solution of urea.....	0.8 ++++	0.7 ++++	0.6 ++++	0.5 ++++
Saturated solution of benzoic acid.....	0.3 —	0.2 —	0.1 —	0.09 —
M/10 tartaric acid.....	0.2 —	0.1 —	0.09 —	0.08 —
1:10 dilution of saturated solution of hydrazin sulfate.....	0.4 —	0.3 —	0.2 —	0.1 —
M/10 acetic acid.....	0.5 —	0.4 —	0.3 —	0.2 —
Acetone, undiluted.....	0.5 —	0.4 —	0.3 —	0.2 —
Ether.....	0.5 —	0.4 —	0.3 —	0.2 —
Chloroform*.....	0.5 —	0.4 —	0.3 —	0.2 —

* Even Tubes 8, 9, and 10 have an excess of chloroform which would eventually hemolyze the blood cells. For accurate quantitative work on red blood cells and complement less

tions of these were carefully made. In the case of uric acid, benzoic acid, and hydrazin sulfate, on account of their slight solubilities, saturated solutions at 37 C. were used. It would perhaps have been better to have made up fractional parts of their molecular solutions based on their solubilities. In dealing with urea and tartaric acid, M/1 and M/10 solutions were carefully made in isotonic salt solution. The antihuman hemolytic system was used, with rabbit complement. Titrations of amboceptor and complement were made daily. One unit of amboceptor and 1 unit of complement were used in the tests for determining the effect of the reagents on amboceptor and complement respectively. Thirty-minute incubations at 37.5 C. in a water bath were used.

Various concentrations of each of the reagents were used in combination with red blood cells and salt solution to determine whether the substance was hemolytic, and if so, in what concentrations. Next, various concentrations were added to tubes containing salt solution and 1 unit of amboceptor, and the whole incubated 30 minutes at 37.5 C. in a water bath. Several volumes of salt solution were then added to bring the concentration below that which of itself would produce hemolysis, red blood cells were added, and the tubes shaken and again incubated to permit of the union of amboceptor and blood cells. The tubes were then removed and centrifuged, and the cells washed several times with salt solution. Enough salt solution plus 1 unit of comple-

TABLE 6—*Continued*
EFFECT OF THE VARIOUS REAGENTS ON HUMAN BLOOD CELLS

Amount (c.c.) Used and Results							
5	6	7	8	9	10	11	12
0.1 +++	0.09 ++++	0.08 ++++	0.05 ++++	0.03 ++++	0.02 ++++	0.01 ++++	0.00 ++++
0.1 —	0.08 —	0.05 —	0.03 —	0.02 +++	0.01 ++++	0.00 ++++	
0.4 ++++	0.3 ++++	0.2 ++++	0.1 ++++	0.08 ++++	0.05 ++++	0.03 ++++	0.00 ++++
0.4 ++++	0.3 ++++	0.2 ++++	0.1 ++++	0.08 ++++	0.05 ++++	0.02 ++++	0.00 ++++
0.08 —	0.06 —	0.04 —	0.03 +	0.02 ++++	0.01 ++++	0.00 ++++	
0.07 —	0.06 +	0.05 ++	0.04 +++	0.03 ++++	0.02 ++++	0.01 ++++	0.00 ++++
0.08 —	0.05 —	0.04 —	0.03 +	0.02 ++++	0.01 ++++	0.00 ++++	
0.1 ±	0.08 +	0.06 ++	0.04 +++	0.03 ++++	0.02 ++++	0.01 ++++	0.00 ++++
0.1 ++++	0.08 ++++	0.06 ++++	0.04 ++++	0.02 ++++	0.01 ++++	0.00 ++++	
0.1 —	0.08 +	0.05 ++++	0.03 ++++	0.02 ++++	0.01 ++++	0.00 ++++	
0.1 —	0.08 —	0.05 —	0.03 ++	0.02 +++	0.01 ++++	0.00 ++++	

than these amounts of chloroform should be used. Blood cells from the author were not hemolyzed in the time mentioned; cells from another individual in the laboratory were.

ment was then added to bring the volume to 1 c.c., and the tubes were replaced in the water bath. In no case was there observed any effect exerted on amboceptor by any of the reagents used.

In testing the effect on complement 1 unit of complement was placed in each test tube along with salt solution, various concentrations of the reagent under investigation added, and the whole incubated 30 minutes. Red blood cells and amboceptor were then added and the tubes re-incubated. Controls were made in which the red blood cells and amboceptor had been permitted to unite before adding the reagents, and also controls were made for the hemolytic system. Data on the hemolytic activity had already been obtained. In the following tables varying degrees of hemolysis are indicated by the symbols used in the Wassermann reaction; a minus sign indicates complete hemolysis, and 4 plus signs indicate absence of hemolysis.

All the reagents included in Table 6, with the exception of uric acid and urea, were able to bring about laking of human red blood cells. Uric acid is a very weak acid, and this together with its very slight solubility may be important factors in eliminating it from the

TABLE 7
EFFECT OF THE VARIOUS REAGENTS ON COMPLEMENT

Reagent	Amount (c.c.) Used and Results			
	1	2	3	4
N/25 lactic acid.....	0.5 —	0.4 —	0.3 +++	0.25 ++++
N/30 HCl.....	0.4 —	0.3 —	0.2 —	0.15 ++
Saturated solution of uric acid.....	0.8 —	0.7 —	0.6 —	0.5 —
M/1 urea.....	0.5 ++++	0.4 ++++	0.3 ++++	0.2 +++
Saturated solution of benzoic acid.....	0.4 —	0.3 —	0.2 —	0.1 ++++
M/10 tartaric acid.....	0.4 —	0.3 —	0.2 —	0.1 —
1:10 dilution of saturated solution of hydrazin sulfate.....	0.4 —	0.3 —	0.2 —	0.15 +
M/10 glacial acetic acid.....	0.5 —	0.4 —	0.3 —	0.2 +
Acetone, undiluted.....	0.2 —	0.15 +	0.10 ++++	0.09 ++++
Ether.....	0.05 ++++	0.04 ++++	0.03 ++++	0.02 +++
Chloroform.....	0.08 ++++	0.02 ++++	0.01 ++++	0.00 —

group of hemolyzers. Urea, while comparatively soluble, is slightly basic in reaction, and apparently does not have an affinity for either the stroma or the hemoglobin of the cell. The group of hemolytic substances mentioned in this table contains mineral acids, fatty acids, aromatic acids, monohydroxy and dihydroxy acids, acetone, ether, chloroform, and hydrazin sulfate.

DISCUSSION

Little is known as to the exact mechanism of hemolysis. Moore¹³ and others, in working with fatty acids and soaps, arrived at the general conclusion that hemolytic agents depend on the presence of a double bond uniting 2 carbon atoms. While this might hold true for the unsaturated fatty acids and soaps, the reagents under discussion in this paper do not fit in with his observations. Mathews,¹⁴ Lilly,¹⁵ and others, mention that ether and chloroform are hemolytic agents, and it

¹⁴ Phys. Chem., 1915, p. 924.

¹⁵ Jour. Exper. Zool., 1914, 16, p. 591.

TABLE 7—*Continued*
EFFECT OF THE VARIOUS REAGENTS ON COMPLEMENT

Amount (c.c.) Used and Results							
5	6	7	8	9	10	11	12
0.2 ++++	0.15 +++	0.1 ±	0.08 —	0.06 —	0.04 —	0.02 —	0.00 —
0.10 ++++	0.09 ++++	0.08 ++++	0.07 +++	0.05 —	0.03 —	0.01 —	0.00 —
0.4 —	0.3 —	0.2 —	0.1 —	0.08 —	0.05 —	0.02 —	0.00 —
0.1 —	0.08 —	0.05 —	0.03 —	0.01 —	0.00 —	—	—
0.09 ++++	0.08 ++++	0.07 ++++	0.06 —	0.05 —	0.04 —	0.02 —	0.00 —
0.08 +++	0.06 ++++	0.05 ++++	0.04 ++++	0.02 ++	0.01 —	0.00 —	—
0.1 ++++	0.09 ++++	0.08 ++++	0.07 +++	0.05 —	0.03 —	0.01 —	0.00 —
0.1 ++++	0.08 ++++	0.06 ++++	0.04 ++++	0.02 +	0.01 —	0.00 —	—
0.08 ++++	0.07 +++	0.06 +	0.05 —	0.03 —	0.02 —	0.01 —	0.00 —
0.01 +	0.00 —	—	—	—	—	—	—

has long been known that mineral acids would act accordingly. So far as known, no report has been made on the remaining substances mentioned in this paper.

Mathews¹⁶ has very clearly summed up the more recent views held as to the way hemolytic agents bring about their results. He states:

"Hemoglobin may be held in the corpuscle by union with the stroma. It is true for all other cells, and probably it is true for the corpuscles, that they are not bags filled with fluid, but they are organized jellies. The corpuscles behave in many ways as if they also were such jellies. Hemoglobin does not escape as one would expect it would if it were in solution, when the corpuscle is punctured or cut across, but it stays in the divided corpuscle. Moreover, when hemoglobin is set free in the corpuscle by some of these methods, particularly in the very large cells of *Necturus*, a tailed amphibian, the hemoglobin may crystallize in the corpuscle itself, which shows that it must be prevented in some way from crystallizing in the normal cell. Moreover, the concentration of hemoglobin in the mammalian corpuscle is greater than the solubility of oxyhemoglobin in an equal bulk of water. For these and other reasons some observers are of the opinion that hemoglobin is held in some kind of a loose chemical or physical union, presumably the former, with the

¹⁶ Phys. Chem., 1915, p. 497.

stroma of the corpuscle and that the various hemolytic agents break this union. It is not at all impossible that the union is with certain reserve valencies of the hemoglobin and the stroma and such unions are very unstable and easily broken."

It is also quite probable that all the reagents do not act in exactly the same way. The color of the supernatant fluid resulting from the action of oxidizing agents, such as the acids, on the red blood cells is greenish probably because of the predominance of biliverdin, whereas the color following hemolysis by chloroform, is more nearly a cherry red, similar to that following the action of immune sera.

It might be worthy of note in discussing the reagents used in this work that the lactic acid was of the racemic form, as is all lactic acid produced synthetically. This differs from the sarcolactic acid found in the body in that it is composed of both d-lactic and l-lactic acid, whereas the latter is d-lactic acid only. There might be some difference, but it is probable, in view of the general hemolytic properties of acids, that it would be a quantitative one only. The racemic form of lactic acid is found in the intestinal tract, and under pathologic conditions, may be present in the stomach. Sarcolactic acid is normally present in the tissues of the body and is especially increased during fatigue. The other hydroxyacid, tartaric, is dihydroxysuccinic acid, found in wine and some fruits, which are probably the source of this acid. Benzoic acid, an aromatic acid, is also taken into the body in fruits and berries. Bacterial decomposition may lead to the formation of phenol carbonic acid (benzoic acid) from the proteins by a series of oxidation reactions. Acetic acid, a fatty acid, is also produced in the intestinal tract as a result of bacterial fermentation. In a typical acidosis there are present aceto-acetic acid, acetone, and β -oxybutyric acid.

Hydrazin sulfate was studied because it was used by Dick¹⁷ to produce necrosis of liver cells in dogs under observation for complement content. This probably breaks up into hydrazin and sulfuric acid. It is quite possible that hemolysis may result from the hydrazin-sulfate molecule, as well as from the action of the dissociation products.

None of these reagents is as strongly hemolytic as the unsaturated fatty acids, which will act in dilutions many times greater than any of the dilutions in which the other reagents mentioned are active.

One point in addition as to the mode of action of these hemolytic agents, is that, if the red blood cells are colloidal jellies, contact with

¹⁷ Jour. Infect. Dis., 1913, 12, p. 111.

free hydrogen ions will permit of a somewhat greater intake of water, and this, according to Brahmachari,¹⁸ materially increases the amount of hemolysis.

An inspection of the table showing the effect of the various reagents on complement shows that all except uric acid were capable of preventing hemolysis. Sachs and Altman¹⁹ (1908), working with unsaturated fatty acids and soaps, showed that these were able to prevent hemolysis by hemolytic sera, and concluded that the action was that of an anticomplement. Moore, Wilson, and Hutchinson, in reviewing this work, speak of the action as a balancing action of hemolyzers; they do not believe that these reagents act as anticomplements. They say in part:

"This result has been stated to be due to the neutralizing of complement, the sodium oleate acting as an anti-complement. We think, however, that there is clear evidence against this view. In the first place, as we shall see later, an *ordinary serum* which is not hemolytic to the corpuscles being used, it may, in fact, be their own serum, is strongly protective against the hemolytic action of sodium oleate. We have followed this question up in detail, as shown by the protocols of our experiments and have successively removed or destroyed (a) immune body, (b) complement, and (c) the lipoids from the active serum. In all cases we have found that no one of these substances is alone responsible for the neutralizing of the hemolytic activity of the sodium oleate. That is to say, in the active serum the immune body and the sodium oleate or linoleate combine and mutually destroy each other's laking power, so that the mixture in due proportion is quite inert upon the blood corpuscles."

The importance of this citation will be evident upon a close inspection of Tables 6 and 7. In the first place urea, which is not a hemolyzer, nevertheless prevents hemolysis by amboceptor and complement. This cannot be a balancing action of hemolyzers. Moreover, of the hemolytic agents which fix complement, the zone of fixation very closely approximates, and in many cases partially coincides with, the weakest dilutions producing hemolysis, and in many cases extends down to include a few which are unable to produce hemolysis. This is a phenomenon very similar to the one mentioned by Moore, Wilson, and Hutchinson. They do not seem to have taken into consideration the fact that the "ordinary serum" they mention as inhibiting the action of their hemolytic agent contained complement. They intimate that complement did take some part in the inhibiting action. After careful investigation of the substances mentioned in Tables 6 and 7, it was finally decided that, while other colloids present in fresh normal serum

¹⁸ Jour. Biol. Chem., 1909, 4, p. 280.

¹⁹ Berl. klin. Wehnschr., 1908, 10, p. 494.

might take some part, the thermolabil colloid called complement played a large part. Whether in the nonhemolytic concentrations that fixed complement the effect was on the midpiece or endpiece of complement, was not investigated. It is a well-known fact that in the Wassermann reaction the midpiece alone is bound, the endpiece remaining free, in the supernatant fluid.

I have shown also that CO_2 prevents complement from combining with amboceptor, but does not prevent amboceptor from combining with the red blood cell. This confirms the work of Sawtschenko,²⁰ who showed that in reality the midpiece unites with amboceptor, but that the endpiece is unable to unite with the midpiece. As soon as the CO_2 is removed, the reaction occurs. As Mathews says, this is probably one of the means the body has of preventing hemolysis in vivo by hemolytic sera.

Hektoen and Ruediger's work with the inorganic salts showed that these salts act as anticomplements in which the ions of Ca and Ba, etc., combine with the complement, giving Ca-complement, Ba-complement, etc.

The molecule of ether is not broken up in the animal body, as is that of chloroform. Graham²¹ showed that in the blood stream chloroform (CHCl_3) is broken up into COCl_2 (Phosgene) and HCl . It would then appear that there are several possibilities for the fixing of complement by chloroform, as all these would possibly be present in the serum removed from an anesthetized animal.

The work of Lillie,¹⁵ McClendon,²² and others, is of interest because it suggests that the anesthetics may, in certain concentrations, play a double rôle in preventing hemolysis by amboceptor and complement. Lillie has observed that anesthetics prevent the outward diffusion of cell pigment produced by salts and thinks this a direct proof that anesthetics prevent increase in cell permeability. In order to determine positively whether the anesthetics were able to destroy or deflect complement in addition to this effect on the cell membrane, the following experiment was made:

One unit of complement (0.1 c.c.), 0.65 c.c. of physiologic salt solution, and 0.05 c.c. of ether were added to each of 3 hemolytic test tubes. These tubes were incubated 15 minutes at 37.5 C. and 0.1 c.c. of a 10% suspension of washed human red blood cells and 1 unit (0.1 c.c.) of antihuman hemolytic

²⁰ Ann. de l'Inst. de Pasteur, 1912, 26, p. 1032.

²¹ Jour. Exper. Med., 1915, 22, p. 48.

²² Am. Jour. Physiol., 1915, 38, p. 173.

amboceptor added. The tubes were again incubated for 15 minutes in the water bath, centrifugated, and the supernatant fluids from all 3 tubes pipetted into a shallow dish, over which a current of air was passed to evaporate the ether. The centrifugated red blood cells in each of the three tubes were washed repeatedly with isotonic salt solution. After the last washing the supernatant fluids were removed and wasted; 0.9 c.c. of physiologic salt solution was then added to Tube 1, and 0.8 c.c. to Tube 2 together with 1 unit of complement; to Tube 3 was added 0.9 c.c. of the original supernatant fluid from which the ether had been evaporated. A 4th tube was then placed in the rack and to it were added 0.1 c.c. of a 10% suspension of washed human red blood cells and 0.9 c.c. of supernatant fluid similar to that added to Tube 3. The tubes were then incubated at 37.5 C. in a water bath for 30 minutes.

The results were no hemolysis in Tubes 1 and 4, and complete hemolysis in Tubes 2 and 3. Hence it is evident that amboceptor had combined with the red blood cells in the presence of the anesthetic, and that all the complement had been deflected but not destroyed. In a future paper a report will be made as to the action of the various reagents on endpiece, midpiece, and the so-called third component of complement mentioned by Ehrlich,²³ Coca,²⁴ and others. These results are of interest in view of the inhibiting effect of ether on phagocytosis in vitro and the lowered resistance following anesthesia as shown by Graham.²⁵ He found that the opsonic power of an etherized serum was restored by evaporating the ether. My work would suggest as an explanation of his results that the ether prevented the complement from combining with the opsonic immune body. The negative results which he obtained for bacteriolysins are not comparable, owing to the necessity of using lower concentrations of ether in these experiments than in those on phagocytosis. They do suggest that in the animal body it is necessary to take into consideration other factors such as acidosis, etc., in addition to the anesthetic itself in order to account for all of the lowered resistance.

If data were available it would be interesting to tabulate for the various secretions, excretions, body fluids, and organ content the amount, if any, of each of these and other reagents present normally, as well as the amount under pathologic conditions, and compare these with the amount necessary to deflect or destroy complement. This might throw light on some acute and chronic infections, as well as on some focal infections. For example, normally the urine contains from 0.005 gm. to 0.02 gm. of urea per cubic centimeter; from Table 2 it may be shown that 0.012 gm. of urea is enough to fix 1 unit of com-

²³ Studies in Immunity, 1906.

²⁴ Ztschr. f. Immunitätsf., 1914, 21, p. 604.

²⁵ Jour. Infect. Dis., 1911, 8, p. 147.

plement. These data might be an explanation of the discrepancy of reports as to the presence or absence of complement in normal urine. It is conceivable that a cystitis might become chronic as a result of the presence of chemical substances interfering with phagocytosis. More recently²⁰ it has been shown that urea is increased in the blood during typhoid fever, especially in the severe cases. Mathews says that under certain pathologic conditions, such as interference with kidney function, the urea may increase to such an extent as to crystallize out on the skin on evaporation of perspiration. Urea may also be increased materially in the saliva, and particularly in the secretions of the duodenum and of the intestine.

Uric acid under normal conditions is present in about 0.00003 gm. per cubic centimeter of blood; under pathologic conditions this may be increased to 0.00015 gm. per cubic centimeter of blood. The amount present in the maximal amount of saturated solution used was only 0.000025 gm., which is one-sixth the amount which may be present under pathologic conditions. Hence, while complement is not affected by the amount used, it may well be that complement is deflected or destroyed by the same amount present under certain pathologic conditions.

From these data it is also clear how hyperacidity of the stomach may favor a chronic infection of the stomach. The same may be true for the intestinal tract in general.

It is also conceivable that an accumulation of by-products of metabolism in any organ or tissue may similarly lower the resistance of that organ or tissue so as to lead to a localization of infection there. On the other hand, it was noticed that many of the reagents studied seemed to hasten the hemolytic reaction when the concentrations used were much less than those fixing complement; that is, in many cases these tubes hemolyzed before the controls on the hemolytic system did. Hence it is conceivable that in certain weak concentrations many of these organic compounds may increase the efficiency of the immunity mechanism of the body, while in greater concentrations they may act in the reverse manner. It would be well worth while to work out the effect of as many of these reagents as possible on bacteriolysins, opsonins, etc., and also to determine whether it is possible to get a summation of effects, or whether anything acts as a protective substance, interfering in the body with the possible harmful effect of one or more of these compounds.

²⁰ Jouve-Belmelle, *Progrès Méd.*, 1916, 31, p. 149. Abstracted *Jour. Am. Med. Assn.*, 1916, 67, p. 1189.

SUMMARY AND CONCLUSIONS

An apparent drop in complement often occurs as a result of anesthesia. This, however, is not constant.

The apparent drop probably is the result of the presence of the anesthetic, as well as of a slight increase in acidity.

Sodium bicarbonate and lactic acid injected into rabbits did not cause a drop in complement; if anything, they caused an increase. The lactic acid was probably oxidized over into carbonates by the body, thereafter acting as an alkali.

HCl injected into rabbits caused a marked drop in complement. This is as might be expected, since the body is unable to oxidize the mineral acids.

Acetone injected in relatively large amounts failed to cause a drop in complement.

Carbon dioxid, lactic acid, HCl, urea, benzoic acid, tartaric acid, acetic acid, acetone, ether, chloroform, and hydrazin sulfate deflect or destroy hemolytic complement in certain concentrations.

All these reagents, except urea and CO_2 , bring about laking of human red blood cells in certain concentrations.

In general it may be said that the zone of concentrations fixing complement very nearly approximates, and in many cases coincides with, the weakest dilutions producing hemolysis, and may extend down to include a few concentrations which are unable to produce hemolysis.

The amount present in the blood is normally many times less than the amount required to affect complement. Under some pathologic conditions the concentration in the blood may be greatly increased, nearly approaching the amount which would affect complement.

On a rich protein diet the amount of urea normally in urine may exceed the amount necessary to fix complement.

This work suggests the possibility that the power of various organic compounds to deflect or fix complement may be a factor favoring infection or maintaining a chronic infection.

In addition to the selective action of many bacteria for certain tissue as the explanation of their localization therein (Rosenow), there may be also the fact of a lowered resistance of the organ or tissue due to increase of products of metabolism therein.

In very weak concentrations — that is, concentrations much too weak to fix complement — many of these reagents seem to accelerate the hemolytic reaction. This is analogous to the physiologic action of

many pharmacologic products. Acting in this manner they might increase the efficiency of the immunity mechanism of the body.

At least in the case of the anesthetics there is a two-fold mechanism inhibiting hemolysis by amboceptor and complement—the action of the anesthetic on the permeability of the membrane, and the deflection of complement.

A saturated solution of uric acid in physiologic salt solution (37 C.) did not hemolyze red blood cells or fix complement. This concentration is however only one-sixth of that found under certain pathologic conditions.

None of these reagents in the concentrations studied prevented amboceptor from combining with red blood cells.

At least in the case of CO_2 , ether, and chloroform, complement was not destroyed; but part or all of it was deflected.

This work suggests the possibility that some organic compounds might occasionally play some rôle in either inhibiting or intensifying the anaphylactic shock, since complement seems to be involved in this reaction.

BLOOD-CULTURE STUDIES ON TYPHUS EXANTHE- MATICUS IN SERBIA, BULGARIA, AND RUSSIA *

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The blood-culture studies which form the basis of the present report were made by us in the Balkans and in Volhynia (Russia) and Galicia during the spring and Winter of 1915-1916. They represent part of the work of an expedition which in June, 1915, left America for Serbia under the auspices of the American Red Cross, for the purpose of carrying on investigations concerning the cause and prevention of typhus fever. For all the expenses of the expedition, including the purchase of a complete laboratory equipment, we are indebted to the Board of Directors of the Mount Sinai Hospital.

The investigations were actually begun in Serbia only after the occupation of the country by the Bulgarians (October 1915) and were subsequently assisted in every possible manner by the Bulgarian and German military authorities. In January, 1916, the work was also started in Volhynia (Russia) by one of us (Baehr) at the invitation of the Austro-Hungarian Government. During the next few months we were therefore able to carry on the investigations independently in different parts of Europe.

The present communication is limited to the results of blood-culture studies. Reports concerning the serologic and epidemiologic investigations and one dealing with the results of the studies on prophylactic immunization against typhus fever will appear in subsequent numbers of this journal.

The cultivation by anaerobic methods, of an organism from the blood of individuals suffering from typhus fever was first reported in 1914 by Plotz.¹ In 1915 an extensive report dealing with the evidence that this organism is the causative factor in typhus fever was published by Plotz, Olitsky, and Baehr.² They reported the isolation of the bacterium from the blood in pure culture in 18 of 34

* Received for publication November 27, 1916. This work was done during the tenure of the Theodor Escherich and George Blumenthal Jr. fellowships in pathology.

¹ Jour. Am. Med. Assn., 1914, 62, p. 1556.

² Jour. Infect. Dis., 1915, 17, p. 1.

cases of the mild type of the disease endemic among the Russian immigrants in New York (so-called Brill's disease³), or 53% of the cases studied, and in 7 cases of the severe European epidemic typhus, or 100% of the cases of this type studied bacteriologically during the febrile period of the disease. Similar blood cultures made on 198 control cases yielded no such organism. In the work which forms the subject of this report, bacteriologic, serologic, and experimental observations were reported which confirmed the opinion as to the etiologic significance of this bacterium in typhus exanthematicus.

Confirmation of these observations by American bacteriologists has thus far been limited by the rarity of typhus fever in this country. For, with the exception of Mexico, typhus fever in America at the present time is restricted almost exclusively to the sporadic cases occurring among the Russian immigrant population of New York. Bernstein⁴ and Blatteis and Lederer⁵ recovered the organism from such cases of typhus fever. During our absence from the country, Gottesman and Klein isolated the organism from over 50% of the cases admitted to the Mount Sinai Hospital. Anderson⁶ succeeded in cultivating *B. typhi-exanthematici* from the blood of guinea-pigs which had been infected with the blood of individuals suffering from typhus fever.

During the Serbian epidemic of 1914-15 Zinsser and Hopkins⁷ were able to recover the organism from only 3 of a large series of typhus cases. But, as explained by Zinsser, the proper ascitic fluid for most of his cultures was not available and the technic necessary for the successful cultivation of the organism was therefore often impossible. More recently, however, Olitsky, Denzer, and Husk,⁸ working in Mexico, reported the successful isolation of *B. typhi-exanthematici* from 8 cases of the severe form of the disease at present epidemic in that country, or 100% of the cases studied bacteriologically.

In Europe, confirmatory reports were published from two different sources. The cultivation of *B. typhi-exanthematici* in Serbia from the blood of individuals suffering from typhus fever was reported by

³ New York Med. Jour., 1898, 67, pp. 48, 77. Am. Jour. Med. Sc., 1910, 139, p. 484; 1911, 162, p. 196.

⁴ Personal communication.

⁵ Long Island Med. Jour., 1916, 10, p. 169.

⁶ Quoted by Zinsser, Jour. Lab. and Clin. Med., 1916, 1, p. 623.

⁷ Jour. Lab. and Clin. Med., 1916, 1, p. 623. Hiss and Zinsser, Textbook of Bacteriology, 1916.

⁸ Jour. Infect. Dis., 1916, 19, p. 811.

Popoff,⁹ and in Volhynia (Russia) by Paneth.¹⁰ Mühlens and Ficker,¹¹ working in Serbia, also were able to recover the organism from the blood of typhus patients and not from control cases, and Ghon in Prague isolated it from the blood of guinea-pigs which had been infected with the blood of typhus patients. Up to the time of our departure from Europe, June 1916, the reports of the last two observers had not yet appeared in print. Popoff isolated the organism in pure culture from the blood of 6 of 10 cases, Paneth in 2 of 5, Mühlens and Ficker in 12 of 20 cases.

BLOOD-CULTURE METHOD

The method followed in making the anaerobic blood culture was the Liborius-Veillon,¹² using the ascitic-fluid glucose agar recommended by Libman as the optimal medium for bacterial cultivation.¹³ The technic employed was briefly described in our previous communication. In view of the fact that some observers have failed to understand the technic, the procedure will be redescribed so as to include some details which were previously omitted.

The Ascitic or Hydrocele Fluid.—The essential ingredient in the medium is ascitic or hydrocele fluid. It must be clear, free of bile or blood pigment, and possessed of a specific gravity of more than 1.015. A filtered ascitic fluid or one which contains a preservative or which has been sterilized by heating should not be used. Pleural fluids are also valueless.

Before use, the ascitic or hydrocele fluid should be tested both aerobically and anaerobically as to its sterility. In view of the possible presence of hemoglobinophilic anaerobes as contaminations in ascitic fluid, it is always advisable in carrying out the anaerobic test to use the ascitic fluid in blood cultures on nontyphus controls. Even if all the tubes remain apparently sterile, these anaerobic control cultures should be observed in a thermostat for not less than 21 days.

Preparation of Medium.—The agar employed was prepared according to modifications described by Canary.¹⁴ The following ingredients are placed in a 4- or 5-liter flask:

Distilled water	3000	c.c.
Liebig's meat extract.....	12	gm.
Sodium chlorid	15	gm.
Peptone (Witte)	30	gm.
Normal sulfuric acid.....	17.5	c.c.
Agar shreds	85	gm.

The flask is placed in an Arnold steam sterilizer for 2 hours, after which the contents are rapidly filtered into a pot through several layers of gauze to remove the coarser impurities. Meanwhile the whites of 8 eggs are thoroughly

⁹ Deutsch. med. Wchnschr., 1916.

¹⁰ Med Klin., 1916.

¹¹ Personal communication.

¹² Quoted by Besson, Technique microbiologique et serotherapie, 1914, p. 105.

¹³ Jour. Med. Research, 1901, 1, p. 84. Bull. Johns Hopkins Hosp., 1906, 17, p. 215. Libman, Celler, and Sophian, paper read before Association of American Physicians, 1914 (not published yet).

¹⁴ Tr. New York Pathol. Soc., 1905, 5, p. 132.

mixed in 150 c.c. of distilled water. The fluid agar is then gradually cooled to 55 C., with constant stirring, and returned to the large flask, which has meanwhile been cleaned. To this the egg mixture is added. The flask is now thoroughly shaken in order to mix the contents and then is placed again in the Arnold steam sterilizer for 2 hours. It is important that the Arnold sterilizer be under full steam—that is, that the interior be at least 100 C. before the flask is introduced. On removing the flask from the Arnold, the egg white should be coagulated into a firm clump and the agar absolutely clear. This clear agar is carefully decanted and filtered through moistened absorbent cotton.

The agar is then titrated in the following manner. About 100 c.c. distilled water is brought to boil in a porcelain dish. It is then removed from the flame, and 5 c.c. of the fluid agar and then a few drops of a 0.5% alcoholic solution of phenolphthalein are added. The hot agar being constantly stirred, a 1:40 normal sodium-hydrate solution is slowly dropped into it until the first faintest evidence of pink appears throughout the mixture. This is the end point. If the ingredients of the agar have previously been properly measured out, the end point in the titration should be reached when 1.8-2.2 c.c. of the 1:40 normal sodium-hydrate solution have been added.

Let us assume that 2 c.c. happens to be the amount needed to neutralize the 5 c.c. of agar. Then in order to neutralize 100 c.c. of agar, 40 c.c. of a 1:40 normal sodium-hydrate solution would be needed. This is equivalent to 1 c.c. of a normal sodium-hydrate solution, so that the acidity of the agar in question may be called 1.

In our blood cultures, agar was never used which had an acidity of less than 0.9 or more than 1.1. For each 0.1 variation, 1 c.c. of a normal sulfuric-acid solution is added to each liter of agar if too alkaline, or 1 c.c. of a normal sodium-hydrate solution if too acid. The agar is then retitrated. It is seldom, however, that the acidity falls outside the proper limits of 0.9-1.1.

After the titration is completed, 2% glucose is added to the fluid agar. Test tubes measuring 20 cm. in length and 2 cm. in diameter are then filled about half full with the medium (about 20 c.c. in each tube) and sterilized once in an autoclave at 10 kg. pressure for 20 minutes or in an Arnold sterilizer for 20 minutes on 3 or 4 successive days.

Technic of Blood Culture.—In making the blood culture, the 2% glucose agar in 8 of these test tubes is reduced to a fluid by boiling and then cooled to 40-42 C. The skin over the antecubital space on the patient's arm is thoroughly disinfected, preferably by alcohol followed by tincture of iodine. A sterile 15-c.c. syringe and needle are used for obtaining the blood from the median basilic or median cephalic veins. The syringe should not be used until it has cooled.

Having obtained a syringe full of blood, it is now important to work very rapidly, otherwise the blood will coagulate or the medium will solidify. The needle is removed from the syringe by means of a forceps, the tip of which has been passed several times through a flame, and the tip of the syringe also flamed off twice very rapidly. The 15 c.c. of blood are then divided among the 8 tubes of glucose agar, each tube receiving approximately 2 c.c. To each tube in turn, ascitic fluid equivalent to at least one third of the volume of the agar (i. e., 6-10 c.c.) is added. The contents, consisting of glucose agar, blood, and ascitic fluid are now poured once or twice into another sterile test tube so as to secure thorough mixing. It is important, in carrying out the mixing, that the introduction of air bubbles into the medium be avoided. After thorough solidification of the mixture, each tube is then covered with

a layer of plain agar, 2 or 3 cm. deep. Finally, in order to prevent drying-out of the medium, it is often advisable to paraffin the cotton stopper of each tube.

Examination of Colonies.—After being placed in a thermostat at 37 C., colonies usually appear in from 7 to 14 days, tho in a few instances they have been observed as early as the 5th day. Colonies may also appear any time up to the 19th day. Tubes which appear to be negative should therefore be observed for at least 3 weeks and should then be opened and carefully examined.

In order to open a tube a circular scratch is made on the glass near the lower part of the tube and the glass is cracked at this point by a slight blow. By pushing the cotton stopper into the tube, the column of agar can be made to slide into a sterile petri dish. If a colony is seen, the surface over it is seared with a hot knife or a hot platinum loop. The agar over the colony is then dug away with the loop until the colony is reached and a small portion of it is used for a Gram stain. The remainder of the colony is subinoculated on one or more slants of 0.5% glucose ascitic-fluid agar, and grown in a Buchner tube half filled with equal parts of pyrogalllic acid and 30% sodium hydrate. The rest of the original blood-agar column is finally cut into thin slices with a hot knife, so that colonies in the interior will not be overlooked.

In making subinoculations it is important that much material (bacteria) be used and that it be thickly smeared on the surface of the slants. This is essential for successful growth, especially in the first few subinoculations.

The slants used by us for subinoculations consisted of agar prepared in the prescribed manner containing 0.5% or 2% glucose and one third by volume of good ascitic fluid. Subcultures were reinoculated every 7-10 days.

MORPHOLOGIC IDENTIFICATION OF THE ORGANISM

The organism recovered by us in the Balkans and in Russia from the blood of individuals with typhus fever was identical in all respects with the one isolated by us from typhus patients in New York, in Russia by Paneth,¹⁰ in Serbia by Popoff,⁹ and in Mexico by Olitsky, Denzer, and Husk.⁸

Colonies.—In our Russian series of blood cultures, the average time which elapsed before colonies first appeared in the culture tubes was between 8 and 9 days. The time varied, however, in the different blood cultures from 5 to 15 days. In our Balkan series, the average time which elapsed between the time when the blood cultures were taken and the time when the colonies became visible was somewhat longer, 10-11 days, the limits being 6-17 days. This greater interval in the Balkan studies is undoubtedly due to the fact that we were unable to observe the cultures daily, so that colonies were often not noted until some days after they had probably reached a visible size. The average time of 8-9 days observed in the Russian studies, in which daily observations of the culture tubes were made, corresponds almost

exactly with the results obtained by us in our American work and by Olitsky, Denzer, and Husk in their Mexican studies.

In 4 blood cultures of the Russian series (Cultures 6, 15, 18, and 25) colonies developed only in the interior of the tubes, and were therefore not visible through the opaque medium. They were discovered therefore only when the tubes were broken open and the medium cut into slices, that is, 24, 25, 26, and 29 days, respectively, after the cultures were made (see Table 3). These four blood cultures have not been included in the average mentioned, for it is impossible to say when the colonies first appeared. In 3 blood cultures we have observed the development of a colony in the center of a blood clot. This was also observed in the New York studies.

The colony first appears as a small opaque spot which in direct light is white in color. During the subsequent 2-3 days it grows rapidly larger, usually assuming a Y shape, and develops a brownish zone of precipitation in the medium immediately about it. The maximal size (5 mm. in its longest diameter) is reached within a few days after the colony first becomes visible; by this time the precipitation zone around it is also well developed. On cross-section, the colony is usually Y-shaped, each limb of the Y being flattened into one plane. In consistency the growth is always exceedingly soft, and the color, especially of the older colonies, is pale brown.

One of the unusual features in the development of the colony is its initial slow rate of growth—the colonies seldom reaching a visible size before the 8th or 9th day—followed by a second period of very rapid growth. In view of the long period before the colony becomes visible, its very rapid growth during the succeeding 2-3 days is rather striking. Apparently the organism must first adapt itself to the artificial medium, but once this adaptation has taken place, growth seems to proceed very rapidly.

It is not unlikely that some of the organisms present in the blood at the time of the culture do not succeed in adapting themselves to the medium and therefore never develop into colonies. To this we shall again have occasion to refer.

Morphology.—In size and general appearance the organism when stained with a simple anilin dye somewhat resembles the influenza bacillus. It is very small and slender, the average length being about a micron. In young cultures the organism is small and uniform in size. In very old subcultures there is often a mixture of variously

sized bacilli, from coccoid forms up to some measuring 2 microns in length. The bacilli are usually straight, tho slightly curved forms occasionally occur. The ends are rounded or slightly pointed, and often show a tendency to fade off. With special stains (pyoktanin acetic-acid Bismarck brown) an occasional organism will show a fine polar body at one end, more rarely at both. There is no definite arrangement of the bacilli; some lie end to end, but just as many lie side by side or at an angle to one another.

Reaction to the Gram Stain.—The organism is gram-positive. In some of our blood cultures, however, smears made directly from the colonies showed only bacilli which were completely decolorized by Gram's method. In other blood cultures, the predominating organism in the colonies was gram-positive with numerous gram-negative bacilli scattered through the field. In subsequent subcultures, the bacilli always became gram-positive.

This observation has been made independently by Olitsky, Denzer, and Husk.⁸ Like them, we have also made the observation that the bacilli seen in typhus-infected lice are decolorized by Gram's method. Da Rocha-Lima¹⁵ in his recent studies found that these gram-negative bacilli are present in enormous number in the stomach wall of typhus-infected lice. Olitsky, Denzer, and Husk succeeded in cultivating the organism from such lice with the anaerobic methods here described, and observed the gram-negative forms gradually become gram-positive in subsequent transplants.

The gram-negative forms of *B. typhi-exanthematici* sometimes seen in the blood-culture colonies, as well as those seen in the stomach of the typhus-infected louse, are exceedingly minute in size, averaging only 0.5 micron in length. In view of our recent blood-culture observations there is a possibility that, just as in the infected louse, the organism may exist in the human body during the disease in this minute gram-negative form.

The gram-negative forms seen occasionally in early cultures from typhus patients and in typhus-infected lice stain distinctly with acid fuchsin and ordinary anilin dyes. They are not to be confounded with the gram-negative forms seen in smears from very old subcultures, which stain very palely and vaguely with fuchsin, and merely represent dead or degenerated bacilli.

Cultural Characteristics.—Because of the limitation of the facilities under which we were forced to work, it was not possible to transplant

¹⁵ Arch. f. Schiffs-u. Tropenhyg., 1916, 20, p. 17.

each strain of *B. typhi-exanthematici* isolated, on all the varieties of media used in our previous studies. The following cultural characteristics, together with the morphologic and serologic observations, were sufficient to determine the identity of the bacilli isolated by us from typhus patients in the Balkans and in Russia with those previously isolated in America.

The organism is an obligate anaerobe. Six of the 43 strains isolated by us we finally succeeded in growing under aerobic conditions, but only after they had been kept on artificial media for 8 or 9 months. These 6 strains are still facultative anaerobes, growing much better under anaerobic conditions than under aerobic. When kept continuously for several generations under aerobic conditions all 6 showed a tendency to die out, only one strain surviving 4 successive transplantations.

The organism grows only on a medium containing ascitic fluid and glucose. Altho all strains have been cultivated artificially for many months, it has not yet been possible to obtain growth on solid medium which does not contain both ascitic fluid and glucose. The optimal medium contains 1 part ascitic fluid to 2 parts of 2% glucose agar. Even on the slants of this medium a very large amount of culture material (at least 1 loopful) must be used in order to obtain growth and it is even advisable to smear the culture material in thick streaks on the surface. It is quite possible that the bacilli in the superficial portion of the thick streaks through their reductase action improve the anaerobic conditions and in this way favor the growth of the organisms in the deeper portions of the streak.

Under the conditions mentioned the subcultures on slants of ascitic-fluid glucose agar incubated in Buchner tubes containing equal parts of pyrogalllic acid and 30% sodium-hydrate solution usually show evidences of growth after about 3 days. The growth is profuse by the 6th or 7th day, and at that time is soft and creamy in character, raised from the surface and of a glistening white color. Very old subcultures may develop a pale-brownish or pale-reddish-brown tint. In the medium itself a diffuse clouding gradually develops as the growth increases — the precipitation phenomenon of Libman.¹³

In stab cultures on the same medium, growth develops in 4 or 5 days if the column of media be layered with petrolatum liquidum or agar. In these cultures the precipitation of the medium can be seen to spread outward from the site of the stab. We have found that if the stab cultures be properly layered and the cotton stoppers paraffined to

prevent drying of the medium, the bacilli will often remain viable for as long as 3 months, even when kept at room temperature.

In a 2% glucose broth exceedingly slight growth may occur under anaerobic conditions in the form of minute flocculi which settle to the bottom of the tube. In glucose broth containing one third by volume of ascitic fluid the anaerobic growth is more marked, but after a few days the proteins of the ascitic fluid begin to come down. In fact, by the 5th or 6th day there is a heavy sediment in the bottom of the broth, which consists almost entirely of precipitated proteins with relatively few bacilli. As Libman¹³ has already shown for some other organisms, this precipitate is chiefly due to the acidification of the medium resulting from the splitting up of glucose by the bacteria. By the 7th or 8th day the precipitate in the ascitic glucose broth has reached its maximum and the bacilli have almost ceased to multiply. By this time the medium, which before inoculation had an acidity of about 0.6 will, when titrated against phenolphthalein, be found to have reached an acidity of 4 or even 5. That is to say, 5 c.c. of a normal sodium-hydrate solution are needed to neutralize 100 c.c. of the broth.

In the previous work it was reported that the organisms fermented not only glucose with acid-production and precipitation, but also maltose, galactose, and inulin. Raffinose, arabinose, saccharose, lactose, dextrin, and mannite were not fermented. For the purpose of identification we inoculated our strains only on ascitic-fluid agar containing Kahlbaum's litmus and either glucose, lactose, or inulin. The results were identical with those obtained with the American and Mexican strains, glucose and inulin being fermented, but not lactose.* Growth was also always profuse on media containing glucose or inulin, but very scanty on a medium containing only lactose.

SEROLOGIC IDENTIFICATION OF THE ORGANISM

Agglutination with Immune Rabbit Serum.—For further purposes of identification agglutination tests were made with some of our strains, immune rabbit serum being used. Altho all the strains so tested were firmly agglutinated up to the maximal titer of the serum, we do not now consider these results of much importance. For it has recently been demonstrated by Olitsky and Bernstein¹⁶ that the immune rabbit serum prepared artificially by the intravenous injection of organisms which have been grown on serum media may give nonspecific results.

* For a method of purifying lactose for bacteriologic use, see Olitsky, Denzer, and Husk.⁸

¹⁶ Jour. Infect. Dis., 1916, 19, p. 213.

This is due to the fact that such an immune serum contains, beside the specific antibody, antibodies developed against the serum (or protein) employed in the medium on which the bacteria have been grown. These serum antibodies give rise to nonspecific agglutinins, precipitins, complement-fixing bodies, and cellular antibodies. Such immune rabbit serum will, for example, agglutinate or precipitate not only the organism against which the animal has been immunized, but also totally different organisms which have merely been grown on the same ascitic-fluid medium.

Agglutination with Serum of Typhus Convalescents.—All the 41 strains isolated by us were agglutinated in high dilutions (the maximum being 1:1600) by the serum of typhus convalescents. For reasons enumerated in a previous paper the microscopic method was used. At times a given convalescent's serum would agglutinate some strains in one or more dilutions higher than other strains. In fact, the relative agglutinability of the different strains varied somewhat with the different sera. The difference was usually insignificant except in the case of strains which had only recently been isolated. These were sometimes inagglutinable by convalescent sera until they had been subinoculated a few times. On the other hand it was often possible to obtain agglutination in high dilutions with the organism obtained directly from colonies in the original blood cultures.

The details of the agglutination studies and their diagnostic significance will be reserved for one of the subsequent papers.

Complement-Fixation Tests.—In these tests (performed by Dr. Peter K. Olitsky) antigens prepared from the strains isolated in the Balkans, as well as from those isolated in Russia, gave strong complement-fixation with convalescent serum. As in the previous work, the antigen was prepared as follows: The growth on agar slants was suspended in distilled water, heated to 60 C. for 1 hour, then autolyzed at 37 C. for 24 hours and finally filtered through a Berkefeld candle (size N — pressure 100 mm. Hg.). The clear filtrate was used as antigen. Bacterial antigens which have not been passed through a Berkefeld filter are often anticomplementary and therefore not serviceable. Never more than one fourth of the anticomplementary unit was employed.

The hemolytic system consisted of antishoop rabbit amboceptor, 5% suspension of sheep cells, and guinea-pig serum (1:10) as complement. The quantities employed were one half of the amounts employed in the

original Wassermann test. The serum used was inactivated at 56 C. for one-half hour and was absolutely nonanticomplementary.

The following table shows the identical results obtained with antigens made from New York, Balkan, and Russian strains (Table 1).

TABLE 1
COMPLEMENT-FIXATION WITH SERUM FROM A NEW YORK CASE OF TYPHUS FEVER

Amount of Serum from Typhus Convalescent (5th day) (c.c.)	Without Antigen	With Antigen		
		New York	Balkan	Russian
0.00.....	0	0	0	0
0.05.....	0	++++	++++	++++
0.10.....	0	++++	++++	++++

0 = no fixation. ++++ = complete fixation.

Table 1 also illustrates the cross-fixation obtained by using antigen prepared from Balkan and Russian strains of *B. typhi-exanthematici*, and serum from a New York case of typhus fever.

GENERAL RESULTS OF THE EUROPEAN STUDIES

In considering the results of the studies, it must be remembered that the work was carried out under exceedingly primitive conditions. In the Balkan series blood cultures were often made on patients several days' journey from our laboratory, and in some instances it was not possible to place the cultures in an incubator until 4 or 5 days after they had been taken. This, in our opinion, was responsible for negative results with certain cultures, which we have nevertheless included in this series, and which have undoubtedly served to lower the actual percentage of positive results (Table 2).

In the Russian series, the blood cultures with a few exceptions were placed in an incubator within 2 or 3 hours after they were taken. Only in one instance, Culture 7, did 24 hours intervene between the time when a blood culture was taken and the time when it was placed in an incubator. Culture 7 was positive.

The ascitic or hydrocele fluid used in blood cultures of both Balkan and Russian series answered all the requisites previously enumerated. In all, 11 ascitic fluids and 3 hydrocele fluids were employed, and the specific gravity of each was more than 1.020. Each fluid was tested anaerobically by taking a blood culture on a control case. The control cultures included typhus convalescents and patients suffering from various febrile conditions, such as relapsing fever, pneumonia, typhoid fever, and erysipelas. The controls were only made in order to deter-

mine the sterility of the ascitic fluid used, for our supplies were limited and we had already reported uniformly negative results in 198 individuals suffering from febrile conditions other than typhus fever.

Results of Balkan Studies.—Forty blood cultures were made in Serbia and Bulgaria on that number of persons suffering from typhus fever (Table 2). In 19 *B. typhi-exanthematici* was recovered, or in 47.5% of the cultures taken. For reasons just stated, this percentage is probably lower than it should have been. When, as in the Russian studies, it was possible to exercise the proper care in the technic, the percentage of positive results was much higher (Table 3).

TABLE 2
RESULTS OF BLOOD CULTURES IN TYPHUS FEVER IN THE BALKANS

Blood Culture	Days Before 1st Colony Appeared	Number of Colonies	Strain
1.....	6	11	S1
2.....	10	3	S2
3.....	No record	No record	S3
4.....	..	0	
5.....	10	1	S4
6.....	..	0	
7.....	..	0	
8.....	..	0	
9.....	..	0	
10.....	17	1	S5
11.....	..	0	
12.....	..	0	
13.....	13	1	S6
14.....	13	3	S7
15.....	..	0	
16.....	11	2	S8
17.....	..	0	
18.....	..	0	
19.....	12	1	S9
20.....	11	3	S10
21.....	10	1	S11
22.....	..	0	
23.....	..	0	
24.....	..	0	
25.....	..	0	
26.....	9	2	S12
27.....	13	3	S13
28.....	..	0	
29.....	7	2	S14
30.....	13	4	S15
31.....	..	0	
32.....	11	1	S16
33.....	13	3	S17
34.....	..	0	
35.....	19	1	S18
36.....	..	0	
37.....	..	0	
38.....	11	1	S19
39.....	..	0	
40.....	..	0	

TABLE 3

RESULTS OF BLOOD CULTURES IN TYPHUS FEVER IN RUSSIA

Case	Blood Culture	Day of Disease	Days Before 1st Colony Appeared	Number of Colonies	Amount of Blood Cultured (c.c.)	Strain
1	1	9	14	1	11	R2
2	2	10	10	2	8	R1
3	3	7	..	0	6	
	4	12	9	2	10	R3
4	5	1	6	168	12	R4
	6	5	25 (?)	1	8	R7
	11	11	7	2	13	R9
5	7	8	6	3	10	R5
6	8	4	..	0	15	
	9	6	8	2	11	R6
	15	11	26 (?)	1	10	R10
	10	6	..	0	9	
7	12	10	..	0	13	
8	16	14	..	0	6	
9	13	6	..	0	13	
	18	11	29 (?)	1	12	R12
10	14	6	9	1183	13	R11
11	17	9	7	1	10	R8
12	19	8	14	2	11	R16
13	21	7	6	1	11	R14
14	20	3	..	0	12	
	23	5	7	1	11	R15
15	22	5	..	0	13	
	24	6	9	2	6	R13
	27	11	..	0	9	
16	25	6	24 (?)	1	12	R18
17	26	2	15	1	12	R19
18	28	9	..	0	8	
	32	12	..	0	12	
19	29	5	..	0	10	
20	31	13	6	2	12	R17
21	30	6	..	0	12	
	33	9	..	0	10	
22	34	2	7	1	10	R20
23	35	5	7	2	10	R22
24	36	7	10	8	10	R21

Results of Russian Studies.—Thirty-six blood cultures were made in Volhynia and Galicia on 24 persons suffering from typhus fever. Twenty-two of the 36 blood cultures, or 61%, were positive. In some individuals, however, as many as 3 cultures were made at various times during the disease. In all, therefore, *B. typhi-exanthematici* was recovered in pure culture from the blood of 19 of the 24 cases of typhus fever, or in 79%.

This percentage is higher than that in the New York typhus, but lower than that in either the Mexican or the severe Balkan 1914-15 type of the disease. For, on comparing the percentage of positive results with the percentage of mortality in the various forms of the disease thus far studied (the Balkan 1915-16 series being omitted for reasons mentioned) the following is noted (Table 4).

TABLE 4

COMPARISON OF PERCENTAGES OF MORTALITY IN VARIOUS FORMS OF TYPHUS FEVER WITH
THE PERCENTAGES OF POSITIVE BLOOD CULTURES

Type of Disease	Mortality Percentage	Number of Cases Studied	Percentage with Positive Blood Cultures
New York typhus (Brill's disease).....	0.2	34	53
Russian typhus.....	5.5	24	79
Mexican typhus.....	20	8	100
Balkan typhus, 1914.....	18-60	7	100

It is apparent from Table 4, that the severer the disease the more readily is *B. typhi-exanthematici* isolated from the blood. This has also been our experience with the individual cases, the blood cultures in the severer cases being almost uniformly positive, in the milder ones often negative. Among the Russian cases, altho all grades of severity were encountered, many were mild, were in fact, clinically indistinguishable from the type endemic in New York, commonly known as Brill's disease.

In the Russian studies we had the unusual opportunity of taking a culture (No. 5) on a patient on the very first day of the disease. This culture was positive, as were also 2 other cultures (Nos. 26 and 34) made on patients on the second day of illness. It is evident, therefore, that the organism is present in the circulating blood from the onset of the disease.

In this regard it is of interest to note that Culture 31, taken during the last 12 hours of a patient's illness, was also positive. Such results were also reported in the New York studies, and in 2 of the cases of the New York series the organisms could still be recovered from the blood 12 and 36 hours, respectively, after the crisis. All other cultures on convalescents made in New York were negative. In addition to those tabulated in Table 3, blood cultures were also made in the Russian studies on 4 cases during the first week of convalescence, with negative results.

Cultures 5 and 14 being omitted for the present, the total number of colonies developing in the remaining 20 positive cultures in the Russian series was 37. Including therefore both negative as well as positive cultures, we find that 37 colonies developed in 346 c.c. of blood. It seems probable that this may not represent the actual number of bacilli in that amount of blood. As previously stated, it is quite possible that some of the bacilli in the blood do not succeed in adapting themselves to the artificial medium and therefore do not develop into

colonies. But comparing these results with those observed in the milder type of the disease (New York typhus) or in the severe forms (Mexico and Balkans, 1914), we note the following (Table 5) :

TABLE 5

COMPARISON OF DIFFERENT FORMS OF TYPHUS FEVER IN RESPECT TO NUMBER OF COLONIES IN BLOOD

Type of Disease	Mortality Percentage	Amount of Blood Cultured (c.c.)	Number of Colonies
New York typhus (Brill's disease).....	0.3	436	34
Russian typhus.....	5.5	346	37
Mexican typhus.....	20	120	34
Balkan typhus (1914).....	18 - 60	51	74

From Table 5 it is apparent that there is a definite relationship between the degree of bacteriemia and the severity of the disease.

The typhus seen in the Balkans during 1915-16 was midway in severity between the Russian and the Mexican, the mortality being 11%. Unfortunately, there are no records of the amount of blood used in this series of cultures, so that it could not be included in the table. There were, however, 44 colonies in 18 positive blood cultures, as compared with 37 in 20 positive cultures in the Russian series and 34 in 11 cultures in the Mexican. Apparently, therefore, the Balkan typhus of 1915-16 belongs, in Table 5, between the Russian and Mexican types of the disease, both as regards degree of bacteriemia and severity of the illness.

The results summarized in Tables 4 and 5 demonstrate that the intensity of the bacteriemia runs parallel to the severity of the disease. This observation has previously been made in our American studies.

BLOOD CULTURES DURING CHILLS

In practically every case of typhus fever seen in Russia, a history of an onset with a chill could be elicited. More rarely the disease was ushered in with only chilly feelings. During the course of the disease chills or chilly feelings were more rarely encountered, and as a rule occurred during the first week of the illness.

It was our good fortune to be able to take blood cultures on 2 patients during chills. The first one (Culture 5) was made during the initial chill at the very onset of the illness. The patient, a girl of 9 years, had been admitted to the quarantine ward of the typhus hospital because of the presence of the disease among the other members

of her family. At the time of her admission, 3 older sisters and a brother were in the second week of their disease, and the mother was in the first week. The father of the family and this little girl were the only members not affected by the disease at the time of her admission. During the next week both remained apparently in good health and their temperatures, taken 3 times a day, were always normal. On the evening of the 18th of January, 9 days after admission to the hospital, the girl complained of a headache and slight chill. Her temperature at this time was normal. On the morning of the 19th she again felt perfectly well and looked well, but her temperature was 37.5 C. (99.5 F.). The slight chill and headache of the previous evening were suspiciously like the prodromal symptoms of typhus fever, so that it was thought advisable to move the patient to the typhus observation ward. In the evening the temperature rose to 38.5 C. (101.2 F.) and there was again a slight headache. On the morning of the 20th the temperature was down to 38 C. (100.4 F.), and the child again looked and felt well. Toward noon, however, there suddenly occurred a chill lasting about 20 minutes, followed by a rise of temperature to 40.2 C. (104.4 F.). The skin became hot, face flushed, tongue coated, and the patient complained of severe frontal headache. From that time on the disease ran the typical course of a case of typhus fever of moderate severity. As is usual in children, there were no mental symptoms. The exanthem first appeared on the evening of the 4th day of the disease and the temperature dropped rather critically to normal on the 15th day. Both clinically and epidemiologically the case was typically typhus fever.

A blood culture was taken during the initial chill, 12 c.c. of blood being used. One hundred and sixty-eight colonies developed in the blood culture, all of which proved to be pure cultures of *B. typhi-exanthematici*. This was the largest number of colonies which we had thus far observed in any blood culture, even in the severest forms of the disease. We had never previously had the opportunity of taking a blood culture during a chill. Using the same medium and ascitic fluid previously employed, 2nd and 3rd cultures were made on the patient on the 5th and 11th days of her disease. Tho both were positive, they contained only the usual number of colonies, that is, 1 and 2 colonies, respectively.

The second patient was a man of about 38 years. He was admitted to the hospital on the evening of the 5th day of the disease with a

temperature of 39.5 C. (103 F.) and a rather widely spread but fresh exanthem. On the following morning his temperature was 39.2 C. (102.2 F.). In the afternoon of the same day (6th day of the disease) he suddenly had an exceedingly severe chill which lasted almost 2 hours, following which his temperature rose to 40.2 C. (104.4 F.). The chill was so severe that toward the end of it the patient looked as if he were going into a collapse. His face was pinched and slightly cyanosed, and his pulse was thready and rapid. From this time on, the disease ran a very severe course. There were marked delirium and prostration. The temperature began to break on the 10th day of the disease and fell to normal on the 13th day. Convalescence was very much delayed, the patient remaining feeble and emaciated for over a month.

A blood culture (No. 14) was taken during the chill, 13 c.c. of blood being cultured in 6 tubes. On the 9th day thereafter all the tubes were found to be uniformly studded with colonies. Eleven hundred and eighty-three colonies developed, all of characteristic appearance but smaller than usual. Twenty of these colonies were subcultured, and proved to be pure cultures of *B. typhi-exanthematici*. This blood culture contained the largest number of colonies thus far observed, 91 colonies to the cubic centimeter.

It is of some importance to note that on the same afternoon that this culture (No. 14) was taken, 2 other blood cultures (Nos. 13 and 15) were taken with the same medium and ascitic fluid. Both were from patients who were also suffering from typhus fever. One was made immediately before the blood culture described, the other immediately after. In Culture 13, 13 c.c. of blood were cultured in 6 tubes; in No. 15, 10 c.c. in 4 tubes. Culture 13 was observed for 26 days and was negative. Blood-Culture 15 was positive, but contained only 1 colony of *B. typhi-exanthematici*. These two cultures may serve as controls for the media used in Culture 14.

SUMMARY

The bacteriologic studies carried out in the Balkans and Russia on 64 individuals with typhus fever confirmed the observation previously made in New York, that *B. typhi-exanthematici* is present in the blood during the febrile period of the disease. Up to date, therefore, this organism has been found in pure culture in the blood of typhus-fever patients in the United States, Mexico, Serbia, Bulgaria, Austria, and Russia.

In Serbia and Bulgaria anaerobic cultures were made in 40 cases, and *B. typhi-exanthematici* isolated from the blood in 19, or 47.5%. Many of the negative cultures had not been incubated properly, and such cultures have undoubtedly materially reduced the actual percentage of positive results.

In Volhynia (Russia) and Galicia positive cultures were obtained in 19 of 24 cases studied bacteriologically, or in 79%.

The organism is present in the blood in typhus fever from the first day of the disease, and can be recovered from the blood during the entire febrile course of the illness.

The intensity of the bacteriemia runs parallel with the severity of the disease.

In 2 instances, blood cultures were made during a chill — in one case at the very onset of the illness — and the blood was found to contain enormous numbers of bacteria.

These observations confirm and strengthen the evidence already presented that *Bacillus typhi-exanthematici* is the etiologic agent in typhus fever.

LEUKOPENIA AND LEUKOCYTOSIS IN RABBITS *

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In view of the renewed interest in therapeutic injections of dead bacteria, a study of the resulting changes in the leukocytes is pertinent. There regularly occurs in the blood, following intravenous injection of foreign proteins, 2 phenomena: first, within a few minutes, a marked reduction of leukocytes, a condition to which Löwit¹ has applied the term leukocytopenia or leukopenia; and second, after several hours, a marked increase of leukocytes, a condition termed hyperleukocytosis. My paper deals with these phenomena as they occur in the blood of rabbits after the injection of certain bacteria.

HISTORICAL REVIEW

The recognition of a condition of leukopenia in the blood by Wyssokowitsch in 1886, and later by Löwit in 1892, gave rise to 2 general theories in explanation thereof: (1) that the diminution of leukocytes is due to an actual destruction of the cells (Löwit), and (2) that it is due to a withdrawal of the leukocytes from the circulating blood into the capillaries of various organs (Goldscheider and Jacobs).² Löwit was led to his conclusion through failure to examine the blood of the various organs and through finding of a few degenerated white blood cells in the circulating blood. His theory received the support of Römer. Goldscheider and Jacobs, who had produced a leukopenia by intravenous injection of spleen, thymus, and bone-marrow extracts, concluded that the diminution of leukocytes was due to a retention of these cells in the capillaries of the lungs, spleen, and liver as the result of a negative chemotactic influence on the leukocytes by the injected substances. Silverman³ considered the withdrawal of the leukocytes from the circulating blood to the capillaries to be the result of a mechanical obstruction in the capillaries caused by swelling of the endothelial cells, this obstruction sifting out the leukocytes and holding them within the organs. Ewing,⁴ however, claimed to have found a similar swollen condition of endothelial cells in sections of normal livers. Bruce⁴ counted the number of leukocytes in a given field in microscopic sections of lung, spleen, and liver, both in normal animals and in tissues taken during leukopenia, and found in the latter condition a great increase of leukocytes. Ewing⁵ found an average of 17,400 leukocytes per c.mm. in sections of normal liver tissue, the blood from the ear at the corresponding time containing 8500 leukocytes, while in a section of liver taken during leukopenia, he counted 40,000 leuko-

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¹ Studien z. Physiol. u. Pathol. d. Blutes v. d. Lymphe, 1892.

² Ztschr. f. klin. Med., 1894, 25, p. 373.

³ Univ. of Penn. Med. Bull., 1904-05, 17, p. 22 (contains a complete review of the literature).

⁴ New York Med. Jour., 1895, 61, p. 257.

⁵ Proc. Roy. Soc., 1894, 55, p. 295.

cytes per c.mm., the blood from the ear vein containing only 3500. These observers and others asserted that the reduction of the leukocytes was due to accumulation of these cells in capillaries of the internal organs, especially those of the lungs, spleen, and liver, and not to disintegration.

The early observations on the effect of protein-injection recorded only the hyperleukocytosis, overlooking entirely the preceding leukopenia. Thus Hirt in 1856 noted an increase in leukocytes following injection of tincture of myrrh; Binz in 1876 injecting ethereal oils, and Pohl injecting numerous other drugs, obtained similar results. Several theories arose to explain the phenomenon. Limbeck's exudative theory assumed leukocytosis to be dependent on the existence of a localized exudation at the point of infection, the larger the exudate the greater the leukocytosis. Löwit's¹ theory assumed that all conditions of leukocytosis are contingent on a preceding destruction of leukocytes, the latter serving to stimulate the formation of new leukocytes. Schulz and Rieder,² failing to find any actual increase of leukocytes during any stage of leukocytosis, explained the phenomenon as a difference in distribution, the leukocytes in hyperleukocytosis being merely withdrawn from the internal organs to the peripheral vessels. Pfeiffer applied the principle of chemotaxis to the production of leukocytosis, and all the later theories are founded on this principle. Ehrlich¹ believed the leukocytosis to be due to an attractive chemotaxis exerted by microorganisms or toxins on a large reserve of neutrophils always present in the bone marrow. The most generally accepted explanation of leukocytosis is that formulated by Goldscheider and Jacobs,² which explains leukopenia and leukocytosis on the basis of a repellent and an attractive chemotaxis, the first causing a retreat of the leukocytes into the capillaries of the internal organs, the second causing an outpouring of these leukocytes, as well as of those newly formed in the bone marrow, into the general circulation. No explanation, however, is advanced for the change from the repellent, to the attractive chemotactic phase.

TECHNIC

Rabbits were selected for this study on account of the ease with which they may be handled and the relative stability of their leukocytes under ordinary conditions. Three series of animals were studied, including 6 apparently normal rabbits, 9 in which examinations of the leukocytes were made during leukopenia, and 6 in which examinations were made during hyperleukocytosis. In every rabbit counts of the leukocytes, both numerical and differential, were made of blood from as many sources as possible, including that which oozed from the cut surface of the parenchyma of the spleen, liver, lung, and bone marrow. In the differential counts at least 100 cells were counted. The blood smears were stained 1 minute in a 1% methyl alcoholic eosin solution, followed by one-half minute in 1% methyl alcoholic methylene-blue solution. All the rabbits were kept without food for 12 hours preceding the injection and during the entire experiment. Ether was the anesthetic, the animals being restrained as little as possible. The bacteria, consisting of typhoid bacilli, streptococci, and staphylococci killed by heating to 60 C., were injected into the ear vein; the quantity injected varied, that of typhoid bacilli from 3½ to 60 million, streptococci from 2 to 60 million, and staphylococci from 20 to 60 million organisms. In the case of several rabbits emulsions of living streptococci were injected. Small pieces of spleen, liver, lung, and bone marrow were taken during each stage, and fixed in saturated alcoholic solution of corrosive sublimate.

¹ Beitrage zur Kennt. d. Leucocytose, 1892.

² Die Anaemie, in Nothnagel's Specielle Path. u. Therapie, 1898.

THE LEUKOCYTES IN NORMAL RABBIT BLOOD

The number of leukocytes in the blood obtained from the general circulation varied in different normal rabbits (Table 1). The number per c.mm. from the ear vein varied from 3300 to 14,650, averaging about 9000. A rather constant finding was the lower count for blood from the left ventricle, as compared with that from the right — a condition probably due to retention of leukocytes in the lung capillaries, either as a normal process or as the result of irritation from the anesthetic. The counts of blood from the liver and lung parenchymata approximated those for the general circulation; the counts of blood from the splenic parenchyma averaged about 3 times those of the circulating blood.

Table 2 presents typical numeric and differential leukocytic counts of blood from various sources in a normal adult rabbit. In most instances a slight increase of leukocytes was found in blood from the veins of the principal organs; in blood from the splenic parenchyma the count was several times that of the peripheral blood, a fact to be explained perhaps by the function and structure of this organ. Care was exercised to avoid pressure on the spleen and other organs while blood was being obtained from the parenchyma. Usually the blood from other sources contained a fairly uniform number of leukocytes. The blood of a normal rabbit contained 20-30% polymorphonuclear cells, 55-74% small lymphocytes, 4-15% large lymphocytes, and 3-10% mast cells.

THE LEUKOCYTES IN LEUKOPENIA

Nine adult rabbits were selected for the study of leukocytes during the leukopenia produced by intravenous injections of bacteria. The quantity injected varied in different rabbits from the smallest dose of $3\frac{1}{2}$ million dead typhoid bacilli to the largest of 60 million dead typhoid bacilli, streptococci, or staphylococci (Table 3).

The onset of leukopenia occurred in most cases within 10 minutes after injection. In Rabbit 33 there was a drop from 5100 to 2200 leukocytes per c.mm. in the peripheral blood within 2 minutes after the injection of 60 million dead typhoid bacilli; the polymorphonuclear elements during this time decreased from 24% to 1%. In Rabbits 22, 4, and 31 the fall in number of leukocytes per c.mm. in the blood of the ear vein amounted to 8250, 11,450, and 9550, respectively. The lowest count observed during this period was 1250 in the blood of the ear vein of Rabbit 22, the original count being 9500.

TABLE 1
LEUKOCYTIC COUNTS OF BLOOD FROM DIFFERENT SOURCES IN NORMAL RABBITS

Rabbit	Ear Vein	Bone Marrow	Splenic Pulp	Splenic Artery	Splenic Vein	Portal Vein
7	3700	3500	39350
8	8350	16250	25350
23	14650	10350	33500	6350	9400	9100
24	13250	32850	10650	10300
25	8100	11750	62800	11600	16400	6200
30	10900	41500	15300

TABLE 2
TOTAL AND DIFFERENTIAL COUNTS OF LEUKOCYTES IN BLOOD FROM VARIOUS SOURCES IN THE ADULT NORMAL RABBIT *

Source of Blood	Total Count	Differential Count							
		Poly-morpho-nuclears		Small Lympho-cytes		Large Lympho-cytes		Mast Cells	
		No.	%	No.	%	No.	%	No.	%
Ear vein.....	14650	2628	18	9636	66	1314	9	1022	7
Bone marrow.....	10350	1449	14	6624	64	1139	11	1138	11
Splenic vein.....	9400	2726	29	5170	55	940	10	564	6
Splenic pulp.....	33500	5025	15	22445	67	5025	15	1005	3
Splenic artery.....	6350	1270	20	4445	70	381	6	254	4
Superior mesenteric vein.....	6350	1588	25	4127	65	317	5	318	5
Superior mesenteric artery.....	6000	780	13	4440	74	360	6	420	7
Liver pulp.....	5250	1365	26	3308	63	262	5	315	6
Right ventricle.....	7150	1073	15	5219	73	286	4	572	8
Left ventricle.....	4950	1188	24	3267	66	248	5	247	5
Hepatic vein.....	11700	1989	17	7839	67	1404	12	468	4
Portal vein.....	9100	1601	11	6734	74	1092	12	273	3

* Rabbit 23, Table 1.

TABLE 3
COUNTS OF LEUKOCYTES OF BLOOD FROM VARIOUS SOURCES DURING LEUKOPENIA PRODUCED BY INJECTION OF BACTERIA

Rabbit and Its Bacterial Injection	Before Injection (Ear Vein)	During Leukopenia (from 2 to 20 min. after Injection)				
		Ear Vein	Bone Marrow	Splenic Artery	Splenic Vein	Splenic Pulp
1 (40 million streptococci).....	14500	5550	30100	130100
4 (60 million staphylococci)....	20400	8950	28100	16650	186400
10 (40 million staphylococci)....	18650	11000	26500	184000
22 (3½ million typhoid bacilli)...	9500	1250	10050	2600	130900
28 (60 million typhoid bacilli)...	13300	6850	8000	5850	69000
29 (60 million typhoid bacilli)...	12000	5800	10750	3400	59500	132100
31 (60 million typhoid bacilli)...	14550	5000	11600	3750	46400
32 (50 million typhoid bacilli)...	8350	5400	30200	5600	6550	74400
33 (60 million typhoid bacilli)...	5100	2200	22550	2400	118800

[illegible]

TABLE 4

TOTAL AND DIFFERENTIAL COUNTS OF LEUKOCYTES IN BLOOD FROM VARIOUS SOURCES DURING LEUKOPENIA PRODUCED BY INTRAVENOUS INJECTION OF BACTERIA *

Source of Blood and Time of Count	Total Count	Differential Count							
		Poly-morpho-nuclears		Small Lympho-cytes		Large Lympho-cytes		Mast Cells	
		No.	%	No.	%	No.	%	No.	%
Left ear vein 20 minutes before injection of 50 million typhoid bacilli.....	8350	1085	13	7098	85	83	1	84	1
Left ear vein 5 minutes after injection.....	5400	756	14	3240	60	324	6	1080	20
Bone marrow.....	30250	7260	24	22385	74	302	1	303	1
Splenic artery.....	5600	56	1	5376	96	112	2	56	1
Splenic pulp.....	74400	14136	19	53568	72	5952	8	744	1
Splenic vein.....	6550	197	3	6232	95	131	2	0	0
10 to 20 minutes after injection									
Superior mesenteric vein.....	5050	101	2	4646	92	50	1	253	5
Superior mesenteric artery.....	4050	0	0	3888	96	122	3	40	1
Liver pulp.....	14450	289	2	13583	94	289	2	289	2
Portal vein.....	12800	512	4	11648	91	384	3	256	2
Lung pulp.....	9100	182	2	8736	96	0	0	182	2
Right ventricle.....	3450	0	0	8112	96	338	4	0	0
Left ventricle.....	6950	278	4	6464	93	139	2	69	1
Superior vena cava.....	8700	87	1	8004	92	348	4	261	3

* Rabbit 32, Table 3.

decrease being synchronous with a drop in the total number of leukocytes. In Rabbit 22, within 15 minutes after the injection, the polymorphonuclear leukocytes dropped from 29% to 0%, and in Rabbit 28 from 22% to 1%; in all rabbits the drop was considerable, tho not always so marked as in the cases specified. The number and proportion of polymorphonuclear leukocytes in blood from the bone marrow were dependent on when during the leukopenia the counts were made; if made early, the proportion was about normal; if late, the proportion as well as the number was much increased. The proportion of poly-

TABLE 5

LEUKOCYTIC COUNTS DURING HYPERLEUKOCYTOSIS PRODUCED BY INTRAVENOUS INJECTION OF BACTERIA

Rabbit and Its Bacterial Injection	Before Injection (Ear Vein)	Count During Hyperleukocytosis				
		Ear Vein	Bone Marrow	Splenic Vein	Splenic Artery	Splenic Pulp
9 (60 million staphylococci).....	5750	17000	15600
16 (40 million staphylococci).....	11000	37450	34000	41850
20 (50 million staphylococci).....	8400	14800	7300	25250
27 (50 million streptococci).....	7150	16050	23950	17850
34 (60 million typhoid bacilli).....	9650	15700	12260	16000	13600	19100
35 (60 million typhoid bacilli).....	9000	24000	19600	91600	16750	73000

morphonuclear elements in splenic blood was approximately that found under normal conditions, but with the enormous increase in the total number of leukocytes in this blood there resulted also a marked increase in the total number of polymorphonuclear elements.

The Condition of the Tissues.—Lung: Appeared normal. Microscopically, there were moderate edema of the capillary endothelial cells and moderate congestion, with an apparent increase in the number of leukocytes. A differential count of leukocytes found in a cross-section of a large vein showed 132 polymorphonuclear, to 15 mononuclear cells.

Liver: Somewhat congested. Smears of the liver parenchyma from a rabbit which had received an injection of living streptococci, contained numerous organisms, both intra- and extracellular.

Spleen: In practically every case markedly distended and bluish from congestion, in some instances twice its normal size. Smears of the pulp from a rabbit which had received an injection of living staphylococci contained numerous organisms.

Bone Marrow: In most instances apparently normal, in a few cases hyperemic. Sections from the tibia and femur revealed a slight increase of the myelocytes with a decrease of fat cells. Phagocytic activity on the part of the giant cells appeared normal.

THE LEUKOCYTES DURING HYPERLEUKOCYTOSIS

Several hours after intravenous injection of bacteria there occurred a marked increase in the number of leukocytes in the blood from all sources (Table 5). The exact time of the increase varied; usually within 3 or 4 hours there was a decided increase, but the acme was frequently not reached until after from 10 to 15 hours. The degree of hyperleukocytosis was not dependent on the number of bacteria injected; Rabbit 9 receiving 60 million dead staphylococci intravenously developed a leukocytosis after 20 minutes, while Rabbit 20 receiving 50 million of the same bacteria did not develop leukocytosis until after 9 hours. The increase was less marked in blood from the splenic parenchyma and bone marrow than was observed during leukopenia.

TABLE 5—Continued

LEUKOCYTIC COUNTS DURING HYPERLEUKOCYTOSIS PRODUCED BY INTRAVENOUS INJECTION OF BACTERIA

Count During Hyperleukocytosis								
Hepatic Vein	Portal Vein	Liver Pulp	Superior Mesenteric Vein	Superior Mesenteric Artery	Right Ventricle	Left Ventricle	Superior Vena Cava	Lung Parenchyma
.....	16450	15750	3500		
.....	26850	25000	19350		
.....	10250	4750	5400		
12400	24250	13800	17000	19900	24600		20150
8350	11200	15000	12150	13950	9700	8400	13650	16250
12950	16350	23100	19000	16950	23200	18000	30800

Distribution of the Leukocytes.—The increase, involving chiefly the polymorphonuclear leukocytes (Table 6), was rather uniformly distributed throughout the vascular system with the exception of the splenic vein, and the splenic and lung parenchymata, where the increase was much more marked; as a result the proportion of the polymorphonuclear cells was greatly increased. The actual numbers of small and large lymphocytes remained approximately normal, with a resulting drop in their proportions. The mast cells were not increased.

The Condition of the Tissues.—Lung: No changes. Moderately congested, with an increase in the number of leukocytes. A differential count of leukocytes in a large vein showed 79 polymorphonuclear, to 27 mononuclear cells.

Spleen and liver only slightly hyperemic.

Bone Marrow: Markedly hyperemic. Actual number and proportion of the polymorphonuclear elements apparently increased. Fat cells decreased. Numerous karyokinetic figures in the myelocytes, the latter being increased.

TABLE 6
DIFFERENTIAL LEUKOCYTIC COUNTS DURING HYPERLEUKOCYTOSIS PRODUCED BY INTRAVENOUS
INJECTION OF BACTERIA *

Source of Blood and Time of Count	Total Count	Differential Count							
		Poly- morpho- nuclears		Small Lympho- cytes		Large Lympho- cytes		Mast Cells	
		No.	%	No.	%	No.	%	No.	%
Left ear vein 2 minutes be- fore injection of 60 million dead typhoid bacilli.....	9000	1350	15	6750	75	1260	14	90	1
Left ear vein 11 hr. after 1st injection and 5 min. before 2nd.....	8700	7221	83	1305	15	87	1	87	1
Left ear vein 13 hr. after 2nd injection of 60 million dead typhoid bacilli.....	24000	18960	79	3840	16	480	2	720	3
Bone marrow.....	19600	14700	75	4704	24	196	1	0	0
Superior mesenteric artery.....	16950	11357	67	4237	25	1017	6	339	2
Superior mesenteric vein.....	19000	12160	64	5890	31	380	2	570	3
14 hr. after 2nd in- jection									
Splenic artery.....	16750	10720	64	5360	32	335	2	335	2
Splenic vein.....	91600	52212	57	35724	39	3664	4	0	0
Splenic pulp.....	73000	26280	36	37960	52	8760	12	0	0
Portal vein.....	16350	10628	65	5395	33	0	0	327	2
Liver pulp.....	23100	14784	64	7623	33	462	2	231	1
Hepatic vein.....	12950	8288	64	4144	32	259	2	259	2
Lung pulp.....	30800	19404	63	8932	29	924	3	1540	5
Right ventricle.....	23200	17632	76	4872	21	464	2	232	1
Left ventricle.....	18000	7560	42	9180	51	540	3	720	4

* Rabbit 35, Table 5.

SUMMARY

In the blood of normal rabbits the leukocytes varied from 3300 to 14,650 per c.mm., with an average of about 9000. Normally, there were fewer per c.mm. in the blood of the left ventricle than in that of

the right. The blood from the normal liver and lung parenchymata contained approximately the same number of leukocytes as the blood in the general circulation, while in that from the splenic parenchyma the number was several times greater.

Following intravenous injection of bacteria there regularly occurred within a few minutes, usually less than 10, a great reduction in the number of leukocytes in the general circulation, chiefly involving the polymorphonuclear cells. The decrease, which amounted to 11,000 cells per c.mm. in some instances, was not dependent on the quantity of bacteria injected. The lowest count recorded was 1250 leukocytes per c.mm. The blood from the bone marrow, liver, and splenic parenchyma contained a greatly increased number of leukocytes, especially the blood from the latter, in which the count overreached 180,000 in several instances. The increase of leukocytes in the blood from these sources consisted largely of polymorphonuclear elements. The blood from the lung parenchyma did not contain an increase either in the number or proportion of the polymorphonuclear leukocytes. Late during the leukopenia, blood from veins of the internal organs and from the left ventricle contained more leukocytes than did that from the corresponding arteries and right ventricle. Sections of liver, spleen, and bone marrow revealed a hyperemic condition, especially marked in the case of the spleen.

From 4 to 15 hours after intravenous injection of bacteria there occurred a great increase of leukocytes in the blood of the general circulation, consisting largely of polymorphonuclear cells. No direct connection was observed between the quantity of bacteria injected and the height of the hyperleukocytosis. Sections of lung, spleen, and liver showed marked hyperemia; of the bone marrow, hyperemia and increased leukoblastic activity.

DISCUSSION

While there is an agreement among investigators concerning the occurrence of leukopenia and hyperleukocytosis after intravenous injection of dead bacteria, still a number of points remain unsettled. That leukopenia occurs is attributed to a withdrawal of leukocytes from the general circulation into the capillaries of certain internal organs. The evidence supporting this contention consists only of the easily demonstrated fact that there is a great diminution of the number of leukocytes in the general circulation, and an increase in the lung,

liver, and spleen during this stage, associated with an edematous condition of the endothelial lining of the capillaries.

If leukopenia is due to a mechanical obstruction, 2 questions arise: First, does the blood in the capillaries of these organs contain an increased number of leukocytes? And second, why is the mechanical sifting-out process confined practically entirely to the polymorphonuclear leukocytes, as demonstrated in this study, wherein the small and large mononuclear leukocytes are shown to have remained practically normal in the blood of the general circulation throughout the leukopenia?

If the leukopenia is due to a mechanical obstruction resulting in a retention of the leukocytes within the capillaries of the internal organs, examination of fresh blood obtained from these capillaries by deep incision into the parenchyma should reveal an increase in the number of leukocytes over that of the general circulating blood and blood obtained from normal organs; that this is actually the case has been demonstrated in this study (Table 3), for counts of blood obtained from the spleen and liver parenchymata, revealed an enormous increase in the number of leukocytes, reaching in the case of the spleen 186,000 per c.mm. It has been claimed that the lung capillaries play the most prominent part in this retention, but the results from this study assign minor importance to this organ in this connection, counts from its blood revealing in most instances little if any increase, tho sections showed some hyperemia and a slight increase.

On the other hand, it is difficult to conceive of a mechanical narrowing of the capillaries which would retard only the polymorphonuclear leukocytes. It has been advanced that the small mononuclear cells because of their smallness are able to pass through the narrowed capillaries, but the advocates of the mechanical theory apparently have not considered the fact that the many thousand polymorphonuclear leukocytes retained in the capillaries would in themselves furnish a mechanical barrier sufficient to produce a noticeable decrease in the small mononuclear cells in the circulating blood. While the blood from the liver and lung parenchymata during leukopenia suffers in part the same drop in polymorphonuclear elements as the peripheral blood, that from the spleen does not; here the actual number of polymorphonuclear cells per c.mm. is greatly increased. The results here adduced show that during the leukopenia the blood in the capillaries of the spleen and to a considerably less degree of the liver and lung, contains not only an increased number of leukocytes, but also an increased proportion of

polymorphonuclear elements; and that the lung does not contain a great increase of these cells, as has been reported. On the other hand, the blood from all sources, that from the splenic parenchyma excepted, contains practically a normal number of mononuclear cells. The mechanical theory, therefore, does not adequately explain the phenomenon of leukopenia.

Goldscheider and Jacobs² explained it on the theory of a negative or repellent chemotaxis, according to which the introduction into the general circulation of a foreign protein repels the polymorphonuclear leukocytes, causing them to find refuge within the capillaries of the internal organs. Certain results in this study furnish reasonable grounds for questioning this explanation.

Smears of the liver and splenic pulp of rabbits, made immediately after the onset of leukopenia produced by injections of living streptococci and staphylococci, revealed numbers of the injected organisms. Blood obtained from the parenchymata of these organs at the same time contained a greatly increased number of leukocytes, especially of the polymorphonuclear type, while the circulating blood contained relatively few cells of this type.

Bull⁸ has demonstrated that organisms injected intravenously into animals disappear from the blood almost completely within 10 minutes after injection, and that many of these organisms can be found in sections of the liver and spleen. It is interesting to note that this period of time corresponds practically with that of the appearance of the leukopenia as found in this study.

While a negative or repellent chemotaxis is theoretically possible and as such might explain the production of leukopenia, still there is some doubt in the opinion of some observers as to the actual occurrence of a negative chemotactic action on leukocytes.^{9, 10}

The evidence adduced here leads to the conclusion that leukopenia, at least in many instances, is not the result of a negative chemotaxis, but rather of a positive chemotaxis, acting on the polymorphonuclear leukocytes, which are chiefly concerned with phagocytosis, attracting them to the spleen and liver, and perhaps other organs, in which the injected or infecting organisms, or their products, have been filtered from the circulating blood; and because the other types of leukocytes are not so vitally concerned in phagocytosis they remain practically in normal numbers in the general circulation.

⁸ Jour. Exper. Med., 1915, 22, p. 475.

⁹ Cited by Wells, Chemical Pathology, 1907, p. 211.

¹⁰ Compt. rend. Soc. de biol., 1912, 72, p. 722.

Whether the leukopenia occurring in typhoid fever, malaria, and other diseases can be explained on these grounds is not determined, but the enlargement of the spleen in these diseases supports, in part, this conclusion.

The striking findings in the spleen during leukopenia, and to a less degree during leukocytosis, have suggested an investigation of the effect of intravenous injection of bacteria in splenectomized rabbits. A report of these results will be made later.

During the stage of hyperleukocytosis many of the polymorphonuclear leukocytes present in the spleen and other organs during leukopenia, are discharged into the general circulation, this being indicated by the high counts of these cells in blood from the splenic and other veins in some cases. In addition the new polymorphonuclear cells produced in the bone marrow are thrown out into the circulation, further augmenting the number and proportion of these cells.

No relation between the height of the leukocytosis and the quantity of bacteria injected could be detected. Goldscheider and Jacobs² claimed that the degree of hyperleukocytosis was dependent on the quantity of protein injected; Lassabliere and Richet,¹⁰ injecting albumin and peptone intraperitoneally, found no relation between the amount injected and the height of the leukocytosis. It was observed in this study that some rabbits would respond to a given dose of bacteria with a higher leukocytosis than others. Probably other, as yet undetermined, factors play an important part in the degree of leukocytosis produced.

CONCLUSIONS

Within a few minutes following the intravenous injection of dead bacteria into rabbits there results a marked decrease in the number of leukocytes in the circulating blood, resulting in a condition of leukopenia.

The decrease in the number of leukocytes involves chiefly the polymorphonuclear elements, in some cases these cells practically disappearing from the circulating blood.

Blood obtained from the splenic and hepatic parenchymata during leukopenia contains an enormously increased number of leukocytes per c.mm., as well as an increased number and proportion of polymorphonuclear elements.

The small and large mononuclear leukocytes remain practically normal in number in the blood from all sources, the splenic parenchyma excepted, during both leukopenia and hyperleukocytosis.

The mast cells may be increased late in leukopenia, just before the onset of hyperleukocytosis.

Blood obtained from the lung parenchyma during leukopenia does not contain an increased number of leukocytes, tho sections during this stage show a slight hyperemia and increase of these cells.

Blood from the bone marrow during leukopenia contains a normal or increased proportion of polymorphonuclear elements, with usually a somewhat increased total number of leukocytes.

Living organisms injected intravenously may be found in the liver and spleen a few minutes after the injection, synchronously with the onset of leukopenia.

A theory of the causation of leukopenia based on a mechanical obstruction to the passage of the leukocytes in the small capillaries of the internal organs does not adequately explain the phenomenon.

A negative chemotactic influence does not adequately explain the production of leukopenia.

The finding, during leukopenia, of injected living bacteria in the spleen and liver, synchronously with a great increase of polymorphonuclear leukocytes in these organs, and a great decrease of these cells in the general circulation, suggests that leukopenia is best explained on the basis of a positive chemotaxis.

Hyperleukocytosis is the result of the discharge from the spleen, liver, and perhaps other organs, of the leukocytes accumulated therein during leukopenia, plus the new cells produced in the bone marrow.

During hyperleukocytosis the blood from all sources in the body shows a great increase in the number and proportion of the polymorphonuclear leukocytes.

HYPERSENSITIVENESS TO TUBERCULIN AS DETERMINED BY INTRACUTANEOUS INJECTION OF DIFFERENT DOSAGES *

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Hypersensitiveness to tuberculin was studied in 28 patients, 13 males and 15 females. Fifteen were 10 years old or younger, 4 were between 11 and 20 years of age, 5 between 21 and 30, and 4 were between 30 and 60 years of age. Nineteen were in the first stage of the disease, 6 in the second stage, and 3 in the third. Eighteen were in the first class of Turban, 7 in the second class, and 3 in the third. Twenty-two were improving, and 6 were stationary. The patients were on my service in the Eagleville Sanatorium for Consumptives, the Home for Consumptives, Chestnut Hill, the pediatric ward of the Jewish Hospital, and in my private practice.

The tuberculin was injected into the skin, as a rule three injections of different strengths being made at one time in the forearm in a diagonal line, so that no injection was directly below another. With a few exceptions that will be referred to later, the smallest injection was given distally, and the largest proximally. Care was of course taken to insure accuracy of dosage.

T. R. (Tuberculin Rückstand) was injected in the case of all but 2 of the patients; the latter were injected with old tuberculin. As a rule the first injections were one ten-millionth, one millionth, and one hundred-thousandth of a milligram. If no reaction was observed, the patient was next injected with one ten-thousandth, one thousandth, and one hundredth of a milligram. If these injections provoked no reaction, the patient was injected with one tenth of a milligram, one milligram, and ten milligrams. The injected areas were examined at the end of 24 hours in every case, and again at the end of 48 hours in most of the cases, and in a number of cases at other intervals up to 36 days. In a few cases the patient tested was examined at intervals of several hours.

The character of the response varied. An areola alone was noted in 12 patients and 17 tests; induration alone in 4 patients and 5 tests; a papule alone in 4 patients and 5 tests; areola and induration together in 14 patients and 34 tests; areola and papule in 19 patients and 37 tests; induration and papule in 2 patients and 3 tests; and areola, induration, and papule in 17 patients and 48 tests. Only when induration or a papule was present was the response regarded as a true reaction.

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VARIATION IN HYPERSENSITIVENESS

The patients varied greatly in their degree of hypersensitiveness, one patient reacting to one ten-millionth of a milligram, and another only to ten milligrams, a dose one hundred million times as great (Table 1). Six patients reacted to one ten-millionth of a milligram, 2 patients to one millionth of a milligram, 7 to one one-hundred-thousandth of a milligram, 2 to one ten-thousandth of a milligram, 1 to one thousandth of a milligram, 1 to one hundredth of a milligram, 3 to one

TABLE 1
THE REACTION IN EACH PATIENT TO THE DIFFERENT DOSES OF TUBERCULIN

Patient	Duration	Milligrams Injected								
		.0000001	.000001	.00001	.0001	.001	.01	.1	1	10
S. G.	2 wk.	+	—	++	?	++	+++			
J. G.	2 wk.	—	—	++	—	+++	+			
C. C.	2 wk.	+	+	+	+	++	++			
Y. D.	2 wk.	+	+	—	+	++	++			
M. C.	2 1/4 mo.	—	—	+	+++	++	+++			
J. P.	2 1/4 mo.	—	—	+	+	+++	+++			
J. Y.	2 1/4 mo.	—	—	++	++	+	—			
R. P.	2 mo.	—	—	++	—	++	+++			
J. B.	2 wk.	—	—	—	++	+	+++			
D. A.	2 1/4 mo.	—	—	++	++	++	+++			
Y. S.	6 wk.	—	—	—	—	—	—	++	++	
R. G.	2 1/4 mo.	—	+	—	++	++	++			
E. P.	6 wk.	+	+	—	—	+	+	+++	++	
R. B.	2 wk.	+	?	—	+	+	++			
I. A.	2 wk.	?	?	++	++	++	++			
S. K.	1 day	—	+	—	—	—	—			
C. W.	2 wk.	+	+	+	?	?	?			
E. J. M.	1 wk.	—	—	—	?	+	+		
I. P.	1 day	—	—	?	—	—	—	?	
E. J.	1 wk.	—	—	—	—	—	—	?		+++
G. R.	10 days	—	—	—	—	+	—	—	+++	
H. G.	1 day	—	+	+
G. F.	1 day	—	—	—
H. J.	1 day	+	+	—
W. S.	1 day	+	—	—
F. M.	1 day	—	+	—

The letters are the initials of the patients' names, "Duration" refers to the time from the first to the last injection, — = no reaction, + = slight reaction, ++ = moderate reaction, and +++ = marked reaction.

tenth of a milligram, 2 to one milligram, and 1 to ten milligrams. Great irregularity was shown in the reactions. Only 12 of the 28 reacted to all the doses of tuberculin above the one that caused the minimal reaction. The rest failed to react in one or more instances to a dose larger than that which caused a distinct reaction. In 10 of these the injection of both doses was made at the same time. In 5 of the 16, including one of the group just mentioned, the larger dose to which they failed to react was the first of a subsequent series of 3 injections given after a short interval—in some cases 2 weeks, and in one 2 months, after the previous series. Failure to react, therefore, may have been due to

a lessening of the hypersensitiveness during the interval. In 1 patient the first reaction was slight and may have been a false reaction. One patient failed to react at all to a dose of one-hundredth milligram.

In only 1 patient was there any systemic reaction. A woman, 26 years of age, in the third stage and in the third class of Turban, showed no reaction of any sort to doses of one ten-millionth, one millionth, one one-hundred-thousandth, one ten-thousandth, one thousandth, and one hundredth of a milligram. Following a subsequent injection of one-tenth milligram there appeared an areola one-fourth inch in diameter, and following the simultaneous injection of 1 milligram an areola one-half inch in diameter. Following the injection at the same time, however, of 10 milligrams, an abscess developed, which after 3 weeks still contained fluid. The patient developed a fever which persisted and she became progressively worse. Three other patients developed abscesses at the site of inoculation, not due to infection, and resembling the abscesses I have seen occasionally develop after the therapeutic injection of tuberculin and of Sherman's "non-virulent tubercle bacilli vaccine." A boy aged 7 years, an

TABLE 2

REACTIONS IN THE SAME PATIENTS TO THE SAME DOSES, THE LARGEST DOSE ON ONE OCCASION HAVING BEEN INJECTED DISTALLY AND ON ANOTHER PROXIMALLY

Patient	8/23/15			10/16/15		
	Proximal .0000001 mg.	Middle .000001 mg.	Distal .00001 mg.	Distal .0000001 mg.	Middle .000001 mg.	Proximal .00001 mg.
S. G.	—	—	—	+	—	++
J. R.	—	+	+	—	—	++
C. C.	—	?	+	+	+	+
Y. D.	+	+	?	+	+	—

incipient case in Turban's Class 1, had shown very slight induration and no areola to an injection of one hundred-thousandth of a milligram. Ten weeks later he showed a slight papule one-fourth inch in diameter, covered with a slight areola, at the site of the injection of one ten-thousandth of a milligram, and a marked and indurated papule with redness in the center and a slight areola seven-eighths inch in diameter at the point where one-hundredth milligram had been injected simultaneously. This papule was still hard at the end of a month, and most of the abscesses began thus altho no abscess developed here. Between these two papules, however, where one-thousandth milligram had been injected at the same time, a very marked and indurated papule appeared, seven-sixteenths inch in diameter, with marked redness and with a moderate areola seven-eighths inch in diameter. This papule remained hard and indurated for 2 weeks, then became fluctuating, and shortly afterward disappeared, leaving an area of induration that persisted for several weeks. Another boy 9 years old, in the first stage and the first class of Turban, who had not reacted to one ten-thousandth of a milligram, developed small abscesses the size of peas 1 week after the injection at the same time of one thousandth and one hundredth of a milligram, the site of the former having shown a marked and indurated papule and a slight areola, five-eighths inch in diameter, and the latter a slight papule without much induration and a slight areola, three-fourths inch in diameter. A girl aged 12 years, in the first stage and the first class

TABLE 3
THE CHARACTER OF THE REACTION AFTER DIFFERENT INTERVALS

Patient	Milligrams Injected	24 Hours			
		Papule	Induration	Areola	Tender
R. G.001	—	+	++	—
	.01	++	++	+	+
	.1	++	++	++	+++
E. P.000001	—	+	+	+
	.01	—	+	+	+
	.1	—	+++	++	++
S. G.01	+	+	+	—
	.1	+	+	+	—
	1	++	+++	+	—
C. C.00001	+	—	+	—
J. Y.01	—	+	+	—
	.1	+	++	+	—
	1	++	+++	++	—
R. P.001	—	++	+	+
	.01	—	+	+	+
	.1	++	+++	++	+++
J. B.001	—	++	+	+
	.01	—	++	+	++
	.1	+	++	++	+++
D. A.00001	—	+	+	++
	.0001	—	+	+	+
	.001	+	++	+	—
M. C.0001	—	++	++	—
	.001	+	+++	++	—
	.01	—	+	+	++
J. P.001	—	+	+	++
	.01	—	+	+	++
	.1	—	+	+++	+
L. A.00001	++	++	+	—
C. W.0000001	—	—	++	—
	.000001	—	—	++	—
	.00001	—	—	++	—
R. V.000001	++	+	++	—
	.000001	++	+	++	—
	.00001	++	+	++	—
E. G.001	—	—	+	+
	.01	+	+	+++	+
	.1	+	+	+++	+
G. P.000001	—	—	+	—
	.0001	—	—	+	—

TABLE 3—Continued
THE CHARACTER OF THE REACTION AFTER DIFFERENT INTERVALS

48 Hours				15 Days			
Papule	Induration	Areola	Tender	Papule	Induration	Areola	Tender
—	++	++	+	++	++	—	—
++	+	+	+	++	++	—	—
+++	+++	++	+	++++	+++	—	++
—	—	+	—	—	+	—	—
—	—	—	—	—	++	—	—
—	++	++	—	++	+++	—	—
—	—	—	—	—	—	—	—
—	+	—	—	—	—	—	—
—	+	—	—	—	—	—	—
—	+	—	—	—	—	—	—
—	+	++	—	—	—	—	—
—	—	—	—	—	—	—	—
+	—	—	—	—	—	—	—
+	+	—	—	++	++	—	—
—	+++	++	++	—	+	—	—
—	—	++	++	—	+	+	—
++	++	++	++	—	++	+	—
—	—	—	—	—	—	—	—
—	—	—	—	—	+	—	—
+	+	—	—	—	++	—	—
—	—	—	—	—	—	—	—
—	+	—	+	+	+	+	—
+	—	—	—	—	+	—	—
++	+	++	—	—	+	+	—
++	+	—	—	—	+	+	—
—	+	+	—	+	++	++	—
—	—	—	—	++	++	++	—
—	+	+++	++	++	+++	+	—
—	++	++	—	—	—	—	—
+	+	++	—	—	—	—	—
+	+	++	—	—	—	—	—
+	+	++	—	—	—	—	—
+++	+	++	—	—	—	—	—
+++	+	++	—	—	—	—	—
+++	+	++	—	—	—	—	—
—	—	—	—	—	—	—	—
+	+	+	+	—	—	—	—
+	+	+	+	—	—	—	—
—	—	—	—	—	—	—	—
—	—	+	—	—	—	—	—

of Turban, on the day following the injection of one-tenth milligram developed a distinct papule, induration, and areola, the latter measuring 1 inch by $1\frac{3}{8}$ inch, with marked tenderness but no pain. The next day the area was marked, indurated, and tender and surmounted by an acutely inflamed papule, one-eighth inch in diameter. The areola remained 3 days, but the papule became a pustule or abscess; at the end of 2 weeks still showing induration, softening, and tenderness.

THE LYMPHATIC CIRCULATION AS A FACTOR

In order to avoid the possibility of the lymphatics carrying part of a larger dose of tuberculin to the place where a smaller dose was injected, the largest dose was injected proximally and the smallest distally. For the same reason the injections were made in a diagonal line so that no two would lie in the same direct line of lymphatic circulation.

By mistake, however, in the first tests that were made, the smallest dose was injected proximally and the largest dose distally (Table 2). As these patients were 7 weeks later given the same dosages in the reverse order, they should furnish an interesting example of the difference in reaction of 3 doses dependent on their position. The reactions showed such variance, however, in the 4 cases records of which were available, that no conclusion can be drawn.

CHANGES OCCURRING IN THE CHARACTER OF THE INDIVIDUAL REACTION

Another interesting study is furnished by observations of the result of the test at various intervals (Table 3).

One patient who received injections of one ten-millionth, one millionth and one one-hundred-thousandth of a milligram on the one arm showed the same reactions to all. An hour and a half after the injection there was marked erythema; 5 hours after there was an area of erythema 7 or 9 mm. in diameter; $14\frac{1}{2}$ hours after there was a pink areola 5 mm. in diameter; 45 hours after there was a slight elevation and the pink areola was 7 mm. in diameter; and 65 hours after the injection there was a slight areola 1 cm. in diameter with a slight elevation.

Forty reactions in 15 patients were examined both on the day following and on the second day following the injection. The reaction was greater after 48 hours than after 24 hours in 7 cases and 10 tests, less in 12 cases and 25 tests, and the same in 4 cases and 5 tests. I have no notes for the third and fourth days. One patient, who showed

no reaction after 24 hours, showed elevation and a slightly red areola after 5 days. In another case the papule was still hard and raised on the sixth day. I have no notes for the seventh day. A marked reaction was noted on the eighth day in a test which showed nothing after 24 hours and a diminishing reaction on the fifth day. I have then no

TABLE 4

THE DECREASE IN HYPERSENSITIVENESS IN PATIENTS AFTER AN INTERVAL OF SEVERAL MONTHS

Patient	Interval	Milligrams Injected								
		.0000001	.000001	.00001	.0001	.001	.01	.1	1	10
S. G.	5½ mo.	+	—	++	?	++	+++ +	+	+++	
J. R.	5 mo.	—	—	++ ++	— +	+++ ++	+			
C. C.	5 mo.	+	+ ++	+ ?	+ ++	++	++			
Y. D.	5 mo.	+	+	—	+	++	++ +	++	++	
M. C.	2¼ mo.	—	—	+	+++ ++	++ ++	+++ +			
J. P.	2¼ mo.	—	—	+	+	+++ ?	+++ +	+		
J. Y.	2¼ mo.	—	—	++	++	+	— ?	++	+++	
R. P.	2¾ mo.	—	—	++	—	++ ++	+++ +	+++		
J. B.	2¼ mo.	—	—	—	++	+ ++	+++ ++	++		
D. A.	2¼ mo.	—	—	++ +	++ +	++ ++	+++			
Y. S.	4 mo.	—	—	—	—	—	— ?	++ +	++ +++	
R. G.	2¼ mo.	—	+	—	++	++ +	++ +++	++		
E. P.	3½ mo.	+	+	—	—	+ +	+ +	+++ ++	++	
R. B.	5 mo.	+	?	—	+ ++	+ ?	++ ++			
I. A.	5 mo.	?	?	++	++ ++	++ ++	++ ++			

records until the fifteenth day, when the reaction was observed in 9 patients and 27 tests. The reaction was greater 15 days after the test than it was on the day after in 3 cases and 7 tests, and greater than on the second day after the test in 7 cases and 13 tests. It was less than on the day after the test in 8 cases and 16 tests, and less also than on

the second day after the injection in 7 cases and 8 tests. It was the same as on the day following the injection in 5 cases and 5 tests, and it was the same as on the second day after injection in 4 cases and 5 tests. A tender lump was noted on the sixteenth day in one patient, and an abscess on the eighteenth day in another. Five patients were observed on the twenty-second day after injection. In one a scar and induration marked the places where on the day following the injections there had been only a slight papule and a slight induration respectively. A slight papule and marked induration were seen in another patient where on the first day there had been slight induration at one spot and a negative reaction at another. Two showed nothing where there had been very slight induration. One showed no induration where there

TABLE 5
THE ALTERATION IN TUBERCULIN HYPERSENSITIVENESS FOLLOWING A COURSE OF TUBERCULIN TREATMENT*

Patient	Date	Intradermal Test in Milligrams	Date	Dose of Tuberculin in Milligrams
S. G.	10/16/15	.00001	11/ 1/15	.00001
J. R.	10/16/15	.00001†	11/ 1/15	.00001
C. C.	10/16/15	.0000001†	11/ 1/15	.00000001
Y. D.	10/16/15	.00000001	11/ 1/15	.00001
M. C.	11/27/15	.00001	12/ 5/15	.00001
J. P.	11/27/15	.00001	1/ 2/16	.00001
J. Y.	11/27/15	.00001	12/ 5/15	.00001
R. P.	11/27/15	.00001	12/ 5/15	.00001
J. B.	2/ 5/16	.0001	2/20/16	.0001
D. A.	11/27/15	.00001	12/ 5/15	.00001
R. G.	11/27/15	.000001	12/ 5/15	.00001
R. B.	10/16/15	.0000001	11/ 1/15	.00001
I. A.	10/16/15	.00001	10/30/15	.00001
Y. S.	11/27/15	.1	12/ 5/15	.01
E. P.	10/30/15	.001	12/ 5/15	.01

* A comparison of the dose of tuberculin administered therapeutically by mouth with that producing a reaction both before its administration and again several months later during the course of tuberculin treatment; and the effect of the tuberculin given therapeutically in reducing the temperature and producing general benefit.

† Smallest amount tested.

had been very slight induration at one place and fairly marked induration at another. One showed slight induration where the induration had been marked. One exhibited a papule and marked induration where there had been a very slight papule and marked induration, and also where there had been no papule but marked induration. A papule was still palpable in one case on the twenty-third day. Induration following an abscess was present on the twenty-eighth day in one patient. On the thirty-sixth day in another patient discoloration, papule, and induration were observed where only slight induration had been noted on the day following the injection.

DECREASE IN HYPERSENSITIVENESS

Fifteen patients were again tested from $2\frac{1}{4}$ to $5\frac{1}{2}$ months after the degree of hypersensitiveness had been first determined. Five reacted in about the same manner as before. Four reacted to 10 times the previous minimal reaction dose, 3 to 100 times it, 1 to more than 100 times it, and 1 to 1,000 times it, while 1 reacted to one hundredth of it.

RELATION BETWEEN TUBERCULIN HYPERSENSITIVENESS AND
TUBERCULIN TOLERANCE

In these 15 cases it was also possible to study the relation between tuberculin hypersensitiveness and tuberculin tolerance. Nine patients

TABLE 5—*Continued*

THE ALTERATION IN TUBERCULIN HYPERSENSITIVENESS FOLLOWING A COURSE OF TUBERCULIN TREATMENT*

Benefit	Reduction of Temperature	Date	Dose of Tuberculin in Milligrams	Benefit	Reduction of Temperature	Date	Intradermal Test in Milligrams
+	—	4/15/16	.05	+	—	4/15/16	.01†
—	—	3/25/16	.01	+	—	3/25/16	.00001†
+	—	3/25/16	.00000001	+	—	3/25/16	.000001†
+	?	3/25/16	.2	+	?	3/25/16	.01†
+	?	4/15/16	.0005	+	?	4/15/16	.0001†
+	—	4/15/16	.1	+	?	4/15/16	.01
+	..	4/15/16	.03	+	—	4/15/16	.1
—	—	4/15/16	.03	+	—	4/15/16	.001†
?	—	4/15/16	.01	—	—	4/15/16	.001†
+	—	4/15/16	.0002	+	—	4/15/16	.00001†
+	—	4/15/16	.01	+	—	4/15/16	.001†
?	—	3/25/16	.0003	?	?	3/25/16	.0001†
—	+	3/25/16	.01	+	—	3/25/16	.0001†
+	—	3/25/16	.3	+	—	3/25/16	.1†
..	—	4/15/16	.1	+	+	4/15/16	.001

were given by mouth as an initial therapeutic dose the exact amount that produced the minimal intracutaneous reaction, 2 were given one tenth of this amount, 2 ten times it, and 2 one hundred times the amount. The initial dose was one one-hundred-millionth of a milligram in 1 case, one one-hundred-thousandth of a milligram in 11 cases, one ten-thousandth of a milligram in 1 case, and one hundredth of a milligram in 2 cases. In no case was the initial dose followed by either a favorable or an unfavorable reaction. My method of giving tuberculin is to hold the dose when it is followed by a favorable reaction, to diminish it when it is followed by an unfavorable reaction, and to increase it when it fails to produce any reaction at all. The early course of tuberculin treatment was apparently of benefit in 9 cases, of possible benefit in 2 cases, and of no benefit in 4. It apparently

reduced the temperature in 1 case, reduced it for a time in 2 cases, but had no lasting or definite effect in the others. There were no untoward effects in any case. After a period varying from 2 to 5½ months the therapeutic dose of tuberculin had been increased until it was 10 times the initial dose in 1 case, 20 times it in 1 case, 30 times it in 2 cases, 50 times it in 1 case, 100 times it in 1 case, 1000 times it in 5 cases, 3000 times it in 1 case, 5000 times it in 1 case, and 20,000 times it in 1 case. In 1 case the dose remained the same.

The actual doses were one one-hundred-millionth of a milligram in 1 case, one five-thousandth of a milligram in 1 case, three ten-thousandths of a milligram in 1 case, one two-thousandth of a milligram in

TABLE 6

DEGREE OF TUBERCULIN HYPERSENSITIVENESS ACCORDING TO AGE, SEX, STAGE, AND PROGRESS OF THE DISEASE

	Total	Milligrams Injected								
		.0000001	.000001	.00001	.0001	.001	.01	.1	1	10
1st stage.....	19	5 1*	2	6	1 1* 1†	1	0	0	1	
2nd stage.....	6	0	0	1	1	1†	0	1 1†	1	
3rd stage.....	3	0	0	0	1	0	1	0	0	1
Male.....	13	1	1	3	3	1	1	1†	2	
Female.....	15	4 1*	1	4	1* 1†	0 1†	0	1	0	1
10 yr. and under.	15	5 1*	0	5	1 1* 1†	0	0	1		
11 to 20 yr.	4	0	2	2						
21 to 30 yr.	5	0	0	0	0	1 1†	1	0	1	1
31 to 40 yr.	1	1	
41 to 50 yr.	1	1†		
51 to 60 yr.	1		
61 to 70 yr.	1		
Improving.....	22	5 1*	2	7	1 1* 1†	1 1†	0	1	1	0
Stationary.....	6	0	0	0	2	0	1	1†	1	1

* Did not react to a smaller dose.

† Tested with O.T. (all others tested with T.R.).

1 case, one hundredth of a milligram in 5 cases, three hundredths of a milligram in 2 cases, a twentieth milligram in 1 case, a tenth milligram in 1 case, a fifth milligram in 1 case, and three-tenths milligram in 1 case. These doses caused favorable reactions in 3 patients and unfavorable reactions in none; they apparently produced beneficial results in 13, possibly in 1, and were of no benefit in 1; they apparently depressed the temperature in 7 patients and possibly in 4 (and not at all in 4 others); they caused harmful effects in none. The intracutaneous test made at this time showed a striking correspondence between the dose clinically determined as the appropriate one and that giving a

minimal reaction. The latter was the same in 1 case, less than a tenth of the therapeutic dose in 5 cases, one tenth in 2, between a tenth and a hundredth in 3, a hundredth in 2, a thousandth in 1 and a hundred times it in another.

A study of the minimal reactions in the patients grouped according to age, sex, stage of disease, and progress reveals certain tendencies: a greater degree of hypersensitiveness seemed to be present in the young, in females, in the first stage, and in improving cases.

SUMMARY

Intracutaneous tests can be made with tuberculin T. R. just as well as with O. T., the preparation usually employed.

The character of the reaction to an intracutaneous injection varies. Reactions most commonly met with, in the order of their frequency, are: areola, induration, and papule; areola and papule; areola and induration; and last areola alone.

Reaction can follow so small a dose as 0.000,000,1 mg., or may not occur until the dose reaches 10 mg., the degree of hypersensitiveness therefore varying 100,000,000 times.

Despite the size of the doses, systemic reaction occurred in only 1 patient, and abscess at the site of inoculation in only 4, the latter probably not being due to infection.

The reaction was greater after 48 hours than after 24 hours in one fourth of the tests; still present after 2 weeks in three fourths of the tests studied, in a number of instances being more marked than after 24 or 48 hours; still present after 3 weeks in 4 of 6 cases studied, in some instances being more marked than after 24 hours; and still present after 5 weeks in 1 case.

Tuberculin hypersensitiveness seems to correspond with tuberculin tolerance. The appropriate therapeutic dose of tuberculin is the dose that gives the minimal reaction when injected intracutaneously. This applies equally well to the initial dose and to any subsequent dose.

The appropriate therapeutic dose as determined by intracutaneous injection is approximated clinically by increasing the dose until a favorable systemic reaction is produced, then maintaining the dose producing this until it no longer produces such reaction, and then again increasing it.

The therapeutic value of tuberculin, properly administered, can be seen in the accompanying decrease in hypersensitiveness.

VIRULENCE AND TOXIN-FORMATION IN B. DIPHTHERIAE *

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INTRODUCTION

The virulence of *B. diphtheriae* and organisms closely related to it morphologically and culturally, is of great importance in the application of quarantine laws. This is especially emphasized by the increasing evidence that many apparently healthy individuals harbor in the nose and throat diphtheria bacilli which are capable of causing an acute attack of diphtheria in nonimmune individuals. Kolmer and Moshage¹ state that 5-20% of healthy individuals harbor the bacilli in the throat, and 10-20% carry them in the nose. In 30-80% of such individuals giving no history of diphtheria, the cultures are avirulent. In individuals who have come into intimate contact with diphtheria cases or have had the disease themselves, the bacilli are virulent in 61-100% of cases. It has been realized for many years that the ability of certain strains of *B. diphtheriae* to produce potent toxins on suitable fluid media does not always run parallel with the virulence of such organisms. This observation has been made especially in connection with Park's No. 8 strain of the diphtheria bacillus, which is in common use in antitoxin laboratories and which produces a potent toxin. This particular strain was isolated about 20 years ago by Dr. Park from a rather mild clinical case of diphtheria. This fact alone would not be sufficient basis for ascribing a low grade of virulence to the strain; for virulence is not dependent on one factor, but on many. Since, however, the strain has retained its strongly toxigenic properties on artificial media for 20 years and at the same time has shown slight virulence for the guinea-pig, it is evident that the toxin-producing powers of this strain cannot be taken as indicative of virulence. The work presented in this paper was undertaken to discover, if possible, some of the relations between toxin-formation and virulence.

Virulence as usually conceived is the disease-producing power of an organism, and is dependent in general on 2 factors—the organism's

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¹ Jour. Infect. Dis., 1916, 19, p. 1.

ability to multiply and produce a toxin and its invasive power. The virulence of an organism, then, may be modified by changing its power to produce toxin in the animal host or by any treatment that may influence its power of invasion or of multiplication. Numerous methods have been devised for the accomplishment of this purpose.

The passage of bacteria through a susceptible host has been shown by many investigators to increase its virulence for that host. It does not always follow that its virulence is increased simultaneously for another host. Thus Ohlmacher² passed a nonvirulent diphtheria strain through a guinea-pig and recovered a virulent diphtheria strain, and Salter³ passing the pseudodiphtheria bacillus through canary birds, thereby increased its virulence. Clark⁴ and others have been unable to confirm these results by identical methods. It has been established without question, however, that *M. pneumoniae* increases in virulence on passage through mice. Staphylococci from boils or carbuncles are recognized as being more virulent than are staphylococci which have lived as saprophytes for a long time. The explanation often advanced for this increased virulence is that of prolonged natural selection; that is, in a large number of organisms introduced there are some weak ones which are killed off by the protective mechanism of the invaded host, leaving only the more resistant to survive. By repeated passage a race is finally developed which may possess many times the virulence of the original culture. That animal passage may sometimes have the opposite effect is shown by the passage of smallpox virus through young calves, which reduces its virulence not only for human beings, but for calves as well.

It is interesting to note the possible relation of capsule-formation to virulence. Certain microorganisms, notably *M. pneumoniae*, *B. anthracis*, and some streptococci, show an increasing tendency to capsule-formation following animal passage and a simultaneously increasing virulence. This is apparently a protective measure on the part of the bacteria against the resistant agencies of the host. It has been pointed out by several workers⁵ that the slimy capsulated organisms are not amenable to agglutination, and that they are taken up by the leukocytes less readily than uncapsulated organisms. Even when a distinct capsule is not visible, according to Eisenberg,⁶ the more virulent organisms may show an ectoplasmic hypertrophy comparable to capsule-formation.

The path of entry and the number of organisms introduced undoubtedly play an important part in the establishment of a disease by microorganisms. The path through which the bacteria enter and the place where they find lodgment must furnish suitable environmental and cultural conditions for their existence and multiplication if the organisms are to manifest their virulence. In diseases in which the bacteria invade the blood stream and give rise to a general bacteriemia (such as typhoid fever, anthrax, and plague), the bacterial virulence is undoubtedly due in part to this ability of the specific organism to penetrate into the blood stream. In other diseases, such as diphtheria and

² Jour. Med. Research, 1902, 2, p. 128.

³ Tr. Jenner Inst. Prev. Med., 1899, 1 (2nd series), p. 113.

⁴ Jour. Infect. Dis., 1910, 7, p. 335.

⁵ Shibayama, Centralbl. f. Bakteriöl., I, O., 1905, 38, p. 482. Porges, Wien. klin. Wchnschr., 1905, 18, p. 691. Gruber and Futaki, München. med. Wchnschr., 1906, 53, p. 249.

⁶ Centralbl. f. Bakteriöl., 1908, 45, p. 638.

tetanus, in which the bacterial growth is localized and in which bacteriemia rarely has been shown, the virulence of the organism must be due to a different set of causes. Even when introduced through the most favorable channels, an organism usually virulent may fail to show any pathogenic powers. This may be due to a number of influences including individual resistance to the microorganism and other immune factors. But it may also be due to the quantity of organisms that is introduced. Experiments carried out by Webb, Williams, and Barber⁸ indicate that the number of anthrax bacilli necessary to produce a fatal infection depends on the virulence of the strain. They found that 3-6 anthrax bacilli taken directly from the blood of a dead animal produced fatal results, while 10-25 times as many taken from an old agar culture were harmless. Also, in working with *B. tuberculosis*, they found that tuberculosis could be produced with 20 bacilli of one strain, while 150 were required of another. The initial dosage, in order to cause infection, must be sufficiently large to overcome any slight natural resistance on the part of the host. It is evident that this amount varies inversely with the virulence.

It has been stated by Bail⁹ that some varieties of highly virulent bacteria produce substances, which he terms 'aggressins,' that are responsible for the high degree of virulence possessed. He believes that the germicidal activity of the body fluids in natural immunity has been over-emphasized, and that certain characteristics possessed by different strains of bacteria which render them insusceptible to phagocytosis play an important rôle in the infectivity of the organism. He has shown that the peritoneal fluid from guinea-pigs dying after a fatal injection of typhoid or cholera organisms possesses the power to increase the infectivity of homogeneous strains that would otherwise be harmless. Thus, when otherwise sublethal doses are introduced together with such peritoneal fluid, the organisms produce a fatal infection. Even tho the animal at the same time be injected with a protective bacteriolytic serum, death may yet be produced when the bacteria and peritoneal exudate are introduced together. In short he holds that the bacteria produce protective bodies for themselves, enabling them to multiply in the host by combating its defenses. These antibodies he believes are in the nature of antiopsonins which protect the bacteria from phagocytosis. By injecting the aggressin exudate into animals, Bail was able to produce an antiaggressin which rendered the bacteria defenseless and permitted phagocytosis. Many⁹ believe that these aggressins are nothing more than endotoxins which have a negative chemotactic influence and not a specific action. The 'virulins' of Rosenow¹⁰ are apparently very similar in nature to Bail's 'aggressins.' Rosenow found that freshly isolated cultures of pneumococci were not phagocytatable, but that this property was lost on subsequent subculture. By extraction with salt solution he obtained a substance from virulent strains which he termed 'virulin,' that increased the virulence of avirulent pneumococci by rendering them less phagocytatable. Whatever may be thought regarding the nature of these substances, it is evident that there are bacterial products formed by some varieties of bacteria which do influence their virulence.

Closely related to this production of defensive agents by bacteria is the

⁷ Jour. Med. Research, 1909, 20, p. 1.

⁸ Arch. f. Hyg., 1905, 52, p. 272.

⁹ Wassermann and Citron, Deutsch. med. Wchnschr., 1905, 31, p. 28. Kolle and Wassermann, Deutsch. med. Wchnschr., 1905, 31, p. 1101. Doerr, Centralbl. f. Bakteriöl., I. O., 1906, 41, pp. 497, 593.

¹⁰ Jour. Infect. Dis., 1907, 4, p. 285.

hypothesis of Welch.¹¹ Welch believes that when bacteria are grown in their own immune sera, they develop more resistance to these sera by some mechanism and become more virulent. Experimental evidence in corroboration of this theory has been produced by Walker.¹² By cultivating *B. typhosus* in its immune serum, he destroyed its power of agglutination (made it immune) and increased its virulence. The immune serum in which the bacilli were grown became at the same time less agglutinative and protective against the bacilli.

The diphtheria group of bacilli have been classified (1) on a morphologic basis, (2) on the basis of biometric reactions, and (3) on the basis of results in virulence tests on guinea-pigs. It is recognized that the morphologic appearance of the organism cannot be relied on entirely for classification within the group, even tho it is of fundamental importance.

THE BIOMETRIC REACTIONS OF *B. DIPHTHERIAE*

The biometric reactions of the group have been carefully studied by several workers¹³ with a view to differentiation of *B. diphtheriae*, *B. hofmanni*, *B. xerosis*, and other diphtheroids. All are agreed that true diphtheria bacilli produce acid in dextrose media and usually from glycerin, and that *B. hofmanni* produces little or none. With other sugars a less constant reaction has been observed.

Knapp,¹³ Zinsser,¹⁴ Morse,¹⁵ and others found that in saccharose broth acid was not produced. Martin,¹⁵ however, found that acid was produced, while Kolmer and Moshage¹³ found that a small percentage of cultures from the nose and throat fermented saccharose. The formation of acid with the latter sugar is considered important in differentiating *B. xerosis* from other members of the group. Martin found that in maltose broth acid was not formed but Morse reports a production of acid. Dextrin, according to Morse, is fermented by some strains and not by others, while according to Martin acid is produced from this sugar by all true diphtheria bacilli. In general the conclusions seem to indicate that the monosaccharids are fermentable by true diphtheria bacilli, but that the higher sugars are fermented less characteristically and less uniformly.

The biometric experiments here recorded were made for the purpose of determining the possible relation between virulent and non-virulent types. It was expected that all would ferment dextrose and glycerin, but that there might be a difference in the degree of acidity or in the fermentation of other sugars that would be of differential value.

¹¹ Brit. Med. Jour., 1902, 2, p. 1105.

¹² Jour. Pathol. and Bacteriol., 1902, 8, p. 34.

¹³ Knapp, Jour. Med. Research, 1904, 12, p. 475. Lubenau, Arch. f. Hyg., 1908, 60, pp. 305, 335. Morse, Jour. Infect. Dis., 1912, 11, p. 253. Heine, Jour. Pathol. and Bacteriol., 1913-14, 18, p. 75. Kolmer and Moshage, Jour. Infect. Dis., 1916, 19, p. 1.

¹⁴ Jour. Med. Research, 1907, 17, p. 277.

¹⁵ Ann. de l'Inst. Pasteur, 1898, 12, p. 26.

Little previous work has been done to determine this point. Moshage and Kolmer¹³ state that virulent diphtheria bacilli are more prone to ferment carbohydrates than nonvirulent, and they suggest that acid-production may run parallel to toxin-production. Goodman¹⁶ found by continued selection of high- and low-acid transfers that from the same original strain low- and high-acid strains could be obtained, and that the virulence for guinea-pigs was much greater in the high-acid strains than in the low. Heine¹⁷ attempted to distinguish virulent from avirulent bacilli by dividing the morphologically true types of bacilli into 3 groups according to their biometric reactions. The first group, or true virulent diphtheria bacilli, ferments dextrose, maltose, lactose, and dextrin, but not saccharose; the second group, often isolated from the skin, ferments dextrose, saccharose, and sometimes maltose, but not dextrin or lactose; and a third group, often found in the urogenital tract, ferments dextrose, maltose, dextrin, and saccharose, but not lactose.

In order to get the optimal conditions for acid-production with a minimal amount of labor, some preliminary studies were made regarding the influence of oxygen tension, of the incubation period, and of certain carbohydrates. They are incorporated in this paper, even tho they do not bear directly on the main theme.

Materials for Study and Methods.—All but 4 of the 26 cultures used in this work were freshly isolated from serum smears taken in clinical cases of diphtheria. The four strains were taken from old stock cultures. In isolating from mixed cultures, it was sometimes possible to transfer from a single isolated colony, but in most instances it was found necessary to transfer some of the mixed culture to fresh Loeffler's blood serum, a small amount of the culture being spread over 3 successive slants without reinoculation of the needle. This was done by side sweeps of the needle from the bottom to the top of the tube. Usually individual colonies of diphtheria bacilli could be fished after 24 hours' incubation and, after determining their purity by strains, transferred to sterile Loeffler's slants. Three colonies could be studied on the same cover slip by employing the technic of Graham Smith.¹⁸ This consists in placing 3 small loops of water on a cover slip, inoculating each from a different colony of bacteria, streaking in parallel lines, drying, fixing, and mounting in Loeffler's methylene blue diluted 1:5. This method has given very satisfactory, rapid results. When a colony was found containing a pure culture of *B. diphtheriae*, a transfer was made from it to Loeffler's blood serum. After 24 and 48 hours' incubation the culture was studied in methylene blue stain for the types of bacteria present (Wesbrook's classification). It was then transferred to 1% glycerin agar, litmus milk, and gelatin for further confirmation before being tested biometrically on 1% dextrose, maltose, saccharose, dextrin, and glycerin broths.

Relation of Oxygen to Acid-Production.—The first series of experiments was carried on in small 60-c.c. Erlenmeyer flasks, 1% dextrose broth with an initial reaction of +0.5 being used. To one series of flasks 5 c.c. of broth were added and to the other series 30 c.c., making a depth of 3-5 mm. and an exposed surface of 17.5 sq. cm. in the first instance, and a depth of 2 cm.

¹⁶ Jour. Infect. Dis., 1908, 5, p. 421.

¹⁷ Jour. Pathol. and Bacteriol., 1913-14, 18, p. 75.

¹⁸ Nuttall and Graham Smith, The Bacteriology of Diphtheria, 1908.

with an exposed surface of 14.4 sq. cm. in the latter. These were sterilized in the Arnold sterilizer and inoculated at the same time from vigorously growing broth cultures of Strain 0 and Park's Strain 8, and incubated at 37 C. with uninoculated controls. Titrations were made in duplicate after 6, 12, 24, 48, and 72 hours. An average of the two titrations was taken (unless they varied widely, whereupon other tests were made) and the titer of the control flask, obtained at the same time, was deducted from it. All titrations were made with phenolphthalein as an indicator, the culture having been boiled for 1 minute in a casserole. Previous to titration, the organisms were killed by heating in the Arnold sterilizer for 20 minutes. At the outset it was questionable whether titrations should be made without heating or according to standard methods. It was realized that there was a possibility of losing volatile acids by heating. A number of tests, made by titrating some in the cold and some after boiling for 1 minute, showed very slight differences. This same result was obtained by Moshage and Kolmer,¹⁹ who state that little difference in acidity is to be observed between cultures tested before and those tested after boiling for 2 minutes.

The results, given in Table 1, seem to indicate that a large exposure to the air increases the rate of production of acid. This is especially marked during the first 24 hours, but is still true at 72 hours.

TABLE 1
THE RELATION OF OXYGEN TO ACID-PRODUCTION BY *B. DIPHThERIAE* *

Time (hr.)	Strain 0		Park's Strain 8	
	5 c.c.	30 c.c.	5 c.c.	30 c.c.
6	0.1	0.0		
12	0.6	0.0	2.0	1.2
24	2.5	1.0	4.8	3.6
48	4.2	2.6	4.8	4.0
72	4.7	3.8		

* 1% dextrose broth used. Results in percentages normal acid.

Further tests bearing on the same point were made by employing Nessler tubes, 17 mm. in diameter, containing 10, 20, 30, and 40 c.c. of dextrose broth. These amounts made a depth of broth in the respective tubes of 4.5 cm., 9 cm., 13.5 cm., and 18 cm. respectively, which gave varying conditions of aerobiosis. Two strains were employed for this experiment, Strain 1 and Park's No. 8. With both strains 1% dextrose broth was used with an initial reaction of -0.5 with the first strain, and +0.5 for the other strain. The medium was prepared and titrated with the sugar added and then sterilized in the Arnold sterilizer. The tubes were inoculated from 24-hour vigorously growing broth cultures and incubated at 37 C. Two tubes with controls were titrated on alternate days, after killing the organisms by boiling the culture for 5 minutes on the water bath. The titrations throughout were made as indicated, and the results obtained by averaging the titers of two tubes and deducting the reaction of the sterile control. The purpose in this experiment was to determine the time of maximal acidity in each group of tubes and its relation to the exposure to oxygen of the air. The results are shown in Table 2. Strain 1 showed a greater amount of acid the 2nd day in the tubes containing the smaller amounts of broth, or in those having relatively the greatest amount of exposure

¹⁹ Jour. Infect. Dis., 1916, 19, p. 19.

to the air. The maximal acidity in the tubes containing 10 c.c. was reached on the 8th day and in the tubes containing 20, 30, and 40 c.c. on the 12th day. In Strain 8 the maximum was reached the 6th day in the 10-c.c. tubes, the 8th day in the 30-c.c. tubes, and the 10th day in the 20- and 40-c.c. tubes. While the results obtained on the relation of oxygen to acid-production are admittedly too meager on which to formulate any general principles, it is evident that the greater the exposure to oxygen the more rapid is the production of acid. It is also evident that for each strain there is a maximal acidity which is reached eventually in 1% dextrose broth regardless of the exposure to oxygen.

TABLE 2
TIME OF MAXIMAL ACIDITY IN 1% DEXTROSE BROTH WITH VARYING OXYGEN TENSION

Amount (c.c.)	Days					
	2	4	6	8	10	12
Strain 1. Initial Reaction — .5						
10	2.0	2.45	2.95	3.6†	2.9	3.35
20	1.9	2.65	2.7	3.15	2.9	3.7†
30	1.75	2.75	3.2	3.1	3.05	3.4†
40	1.15	2.45	2.1	3.1	2.9	3.2†
Strain 8. Initial Reaction + .5						
10	2.3	4.4	5.0†	4.6	4.6	
20	2.4	4.7	4.8	4.8	4.9†	
30	3.1	4.6	4.1	5.0†	5.0	
40	3.1	4.9	4.4	4.6	5.0†	

Results in percentages normal acid.

† Maximal acidity.

Time of Maximal Production of Acidity in Various Carbohydrates.—It has been indicated by the work of Morse²⁰ that the maximal acidity produced by *B. diphtheriae* is reached in the different sugars at a rather constant time interval for the various carbohydrates. She found that the greatest acidity in dextrose occurred on the 13th day; in maltose and dextrin, on the 8th or 9th day; and in glycerin, on the 15th or 16th day. In view of the probable effect of aerobiosis on acid-production, it was deemed advisable to obtain further evidence under the conditions which were to be used in future work, relative to the time of maximal acid-production in the various carbohydrates. The experiments were conducted with 1% of the carbohydrates in infusion broth with an initial reaction of +0.5, dextrose, maltose, saccharose, dextrin, and glycerin being employed. Ten cubic centimeters of the sugar broths were placed in tubes with a uniform bore of 16 mm., making a depth of about 5.5 cm. These were sterilized in the Arnold sterilizer, inoculated from a 24-hour broth culture, and incubated at 37 C. Two strains, Nos. 0 and 8, were used. Every day 2 tubes of each sugar from the two strains with controls were titrated, after boiling for 5 minutes to kill the organisms. This was continued daily for 15 days. It will be seen from Table 3 that the results with the two strains did not coincide in all respects. A slight acidity developed in the case of Strain 8 on saccharose broth the 1st day; otherwise no acidity was found, but a marked alkalinity was prevalent throughout. In dextrin a preliminary acidity

²⁰ Jour. Infect. Dis., 1912, 11, p. 253.

was found for 2 and 3 days respectively, followed by an alkaline reaction. This is in accord with Morse's observations. She found that saccharose was never fermented and dextrin only by some strains. In maltose some acid was formed early until the 6th or 8th day and then there followed an alkaline reaction. In dextrose a high acidity was produced rapidly; the reaction then fluctuated between high acidity and the maximum throughout the entire time, the maximum of Strain 0 being reached on the 9th day and of Strain 8 on the 4th day. Acid was formed in glycerin broth rather slowly by both strains and reached a maximum on the 14th day in each strain.

TABLE 3
TIME OF MAXIMAL ACIDITY IN 1% CARBOHYDRATE BROTHS, INITIAL REACTION +0.5

Carbohydrate	Days															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Strain 0																
Dextrose.....	1.0	2.7	2.8	3.3	3.3	3.6	3.5	3.5	4.1†	3.9	3.9	...	4.0	4.0	4.1†	3.8
Maltose.....	0.6	1.4†	0.5	0.0	0.2	0.1	0.1	0.2	0.1	0.0	0.1	...	0.3*	0.1*	0.4*	0.8*
Saccharose.....	0.2*	0.2*	0.5*	0.2*	0.6*	1.1*	1.2*	1.6*	1.5*	2.3*	2.1*	2.2*	1.9*	2.6*
Dextrin.....	0.1	0.6†	0.1	0.3*	0.3*	0.6*	1.3*	0.9*	1.4*	1.8*	1.6*	1.8*	1.8*	2.0*
Glycerin.....	0.1	0.1	0.3	0.2*	0.3	0.4	1.6	1.8	1.8	1.8	3.4	...	3.8	4.2†	4.2	4.0
Strain 8																
Dextrose.....	0.9	1.6	5.4	6.3†	6.3†	6.0	6.0	5.7	5.5	5.5	5.9	6.5†	5.9	6.2	5.7	
Maltose.....	0.3	1.0†	1.0†	0.5	0.3	0.3*	0.4*	0.4	0.1*	1.6*	1.6*	0.9*	1.5*	1.2*	1.2*	
Saccharose.....	0.25†	0.6*	1.0*	1.5*	1.6*	1.8*	2.0*	2.1*	2.1*	2.3*	2.3*	2.9*	2.5*	2.4*	2.1*	
Dextrin.....	0.2†	0.0	0.3*	0.5*	0.5*	1.1*	1.0*	1.4*	1.8*	1.3*	1.9*	1.8*	1.8*	1.7*	1.6*	
Glycerin.....	0.0	...	0.3	0.9	1.9	1.6	2.6	2.3	2.7	3.9	4.4	4.6	4.4	5.2†	4.0	

Results in percentages normal acid.

* Alkalinity.

† Maximal acidity.

From the results obtained it is evident that the time required for the maximal production of acid in the different sugars varies with different strains of diphtheria bacilli. It appears that by using a standard 8-day observation on all of the sugars, as accurate an index of their biometric power would be obtained as by using a different time limit for each sugar as Morse suggests. It also seems probable that some of the discrepancy between the results obtained by different workers on the fermentative action of *B. diphtheriae* on maltose and dextrin may be due to the period of incubation previous to reading of results. The use of litmus as an indicator in Hiss serum water medium would not record as delicate changes in reaction as does the use of phenolphthalein. The amount of carbohydrate added to the broth seems to make little difference, within limits, in the amount of acid formed. A series of tests showed that 0.5% of the various carbohydrates gave as high maximal acidity as 1%, except in the case of glycerin, in which the acidity seemed to develop more slowly.

In view of the preliminary work outlined, it seemed wise to employ, as standards for the biometric tests, tubes 16 mm. in diameter, each containing 10 c.c. of the broth, to which 0.5% of the carbohydrate had been added previous to sterilization in the Arnold sterilizer, and which showed an initial reaction of 0.5. The carbohydrates employed were

dextrose, maltose, saccharose, dextrin, and glycerin. These were inoculated from 24-hour broth cultures and incubated for 8 days before titrations. Titrations were made after boiling the cultures 1 minute; an average of 2 tubes was taken in each case and the titer of the control tube deducted from it for the final result.

In all, 26 strains were tested in this manner. The results are recorded in Table 4. From the summary in Table 5 it will be seen that

TABLE 4
BIOMETRIC RESULTS FOR 26 STRAINS OF MORPHOLOGICALLY TRUE DIPHTHERIA BACILLI

Culture	Dextrose	Maltose	Saccharose	Dextrin	Glycerin
0	3.7	1.5*	1.6*	1.7*	2.4
122	3.9	1.4*	1.4*	0.9*	1.7
905	3.7	0.9*	1.0*	0.2	1.8
668	4.0	0.6*	0.7*	1.2	1.1
461	3.7	1.2	0.3*	0.0	2.0
1290	3.9	0.9	3.4	0.2*	1.9
694	4.4	0.1	0.0	0.1*	1.2
1303	4.3	0.8*	1.2*	0.4*	3.5
1	2.5	1.2	0.0	0.7	0.5
1502	4.1	0.9*	0.8*	1.7*	0.9
168	4.4	0.0	1.3*	0.6	1.4
880	4.4	0.5	1.2*	1.5*	1.1
1353	4.5	1.1*	1.9*	1.7*	0.4
15	3.3	1.2	0.5*	0.0	0.5
540	4.4	1.4*	1.6*	1.5*	1.1
42	4.6	3.3	4.0	1.6	3.7
600	4.0	0.9	1.3*	0.6*	0.5
1293	4.7	0.8*	1.5*	1.1*	0.4
1441	4.6	0.0	1.7*	1.5*	1.3
Park's	4.8	0.5	2.0*	1.3*	3.0
8	4.2	2.3	1.3*	0.3	2.1
49†	4.6	0.2	0.7*	1.0	5.2
50†	3.75	1.1	0.1*	0.6	0.9
2059	4.0	3.4	4.0	0.0	1.9
11	3.7	0.75	0.9*	0.5	0.8
13	3.65	0.7	0.5*	1.5	1.05
20					

Results in percentages normal acid.

* Alkalinity.

† Furnished thru the kindness of Dr. Theobald Smith and described in "Notes on Two 'Atoxic' Strains of Diphtheria Bacilli" by Brown and Smith (Jour. Med. Research, 1914, 20, p. 443).

TABLE 5
SUMMARY OF BIOMETRIC RESULTS FOR 26 STRAINS

Result	Dextrose	Maltose	Saccharose	Dextrin	Glycerin
Producing acid.....	26	15	3	10	26
Producing alkali....	0	9	21	13	0
No change.....	0	2	2	3	0

all strains fermented dextrose—the low and high limits being 2.5% and 4.8% normal acid, respectively. Nineteen of the 26 fell between 3.5 and 4.5%. In maltose 15 formed acid, 2 produced no change, and 9 produced alkalinity. In saccharose 21 produced alkalinity, 2 remained unchanged, and 3 produced acid. It is interesting to note that in the case of those forming acid, a relatively large amount (over 3.4%) was

formed. In dextrin 10 produced acid, 3 produced no change, and 13 produced alkalinity. In glycerin all of the 26 strains produced acid. There was a wide range, however, in the amount of acid formed, from 0.4 to 5.2%. Twenty produced 2% or below, while 6 produced over 2%. From the results obtained with the 26 strains, morphologically true diphtheria bacilli, it is seen that all ferment dextrose and glycerin, the majority do not ferment saccharose, over half ferment maltose, and less than half, dextrin.

VIRULENCE IN *B. DIPHTHERIAE*

The fact that in many cases after recovery from an attack of diphtheria, avirulent organisms are isolated, early led to the belief that through some unknown action of the body fluids the virulence of the organisms was lost. More recently it has been shown that while 10-20% of well individuals harbor in the nose and throat avirulent organisms belonging to the diphtheria group, the larger percentage of typical diphtheria bacilli isolated after recovery from an attack of the disease are virulent. In a study of 2774 children, Perkins, Miller, and Ruh²¹ found that 105, or 4.07%, were carriers of virulent diphtheria bacilli, potentially capable of producing the disease. As was stated in the early part of this paper, virulence may be due either to the ability of the organism to produce a toxin or to its invasive power or to the combined action of these two factors. In some diseases which are characterized by a generalized toxemia, such as diphtheria and tetanus, the virulence of the organism may be largely dependent on its ability to produce a soluble toxin. Since experiments have shown that in many strains of diphtheria bacilli the virulence, as shown by guinea-pig tests, gives little idea of the toxigenic powers of the strain, the reason for this virulence in relatively atoxic strains has been sought. As yet no satisfactory answer has been given. The diligent effort to modify the virulence of members of this group and to determine the relations existing between the various members, tho unsuccessful in these respects, has contributed much to our knowledge of virulence and of methods for its determination. This problem has been attacked from several angles: (1) animal passage, (2) symbiosis, (3) prolonged cultivation on artificial media, (4) heating, (5) drying, and (6) growth with 'aggressins.'

Methods of Conducting Virulence Tests.—Guinea-pigs have been generally adopted for testing the virulence of diphtheria bacilli because these animals

²¹ Jour. Infect. Dis., 1916, 18, p. 608.

seem to be susceptible in about the same degree as man. The methods that have been employed, however, have been various. As public health laboratories are often called on to make virulence tests for release from quarantine of carriers or convalescents, it is important that the tests employed be as delicate and rapid as possible.

Graham Smith¹⁸ grew the pure cultures on sugar-free broth for 48 hours and injected guinea-pigs subcutaneously. Other workers²² grew organisms on 1% dextrose broth with a reaction of -0.5 for 48 hours, the tubes being slanted to give a maximal exposure to the air, and injected the unfiltered growth in amount equivalent to 0.5% of the body weight, subcutaneously into guinea-pigs weighing 250-300 gm. If at the end of 4-6 days the animal was alive, the culture was considered nonvirulent. These workers ascribed the virulence chiefly to the toxin produced. Arms and Wade²³ found by testing the virulence in a manner similar to that of Weston and Kolmer, that different colonies developing from the same case varied in their virulence, and they suggested that a number of colonies be tested before calling the organisms nonvirulent. Following clinical diphtheria the organisms recovered are usually virulent. This was early shown by Stone,²⁴ who found that in 14 of 18 cases giving positive cultures after 3 weeks the bacilli were still virulent, as proved when 1 c.c. of a 48-120-hour sugar-free-broth culture was injected subcutaneously. Similar results have been reported by many other investigators since that time. Morse²⁵ using guinea-pigs weighing 150-250 gm. and injecting 0.5% of their body weight of a 48-hour culture grown on sugar-free broth with a reaction of -0.5 , classed as virulent those strains that killed within 4 days with typical lesions. Zinsser²⁵ tested for virulence by intraperitoneal injections of the growth from a 24-hour agar slant washed down in 5 c.c. sterile broth. One-fourth cubic centimeter of such a suspension was injected for each 100 gm. of weight of the guinea-pig. The use of an emulsion from Loeffler's blood serum for virulence tests is practiced in many public health laboratories. If the virulence is due to the toxin formed in broth, as Weston and Kolmer have suggested, there may conceivably be wide differences in action and especially in rapidity of action, between living cultures washed from agar or serum and those from broth. More recently Kolmer and Moshage¹ made a comparative study of the various methods employed for the determination of virulence. Tests made with 0.1 c.c. of a 72-hour plain-dextrose-broth culture injected intracutaneously did not give as high a proportion of positive results as did the subcutaneous injection when 0.5% of the body weight of the animal was used, and the results were more uncertain to read. In the intracutaneous test, if positive, superficial necrosis was observed in 48-72 hours. The subcutaneous injection of 72-hour serum-broth cultures gave results as good as those from subcutaneous injection of washed down Loeffler media, but the latter accomplished the results in shorter time. The subcutaneous injection of 72-hour plain-dextrose-broth cultures proved superior to the intraperitoneal injection of 24-hour and 72-hour plain-dextrose-broth cultures. They concluded that when the time required and delicacy of action were considered, the subcutaneous injection of 0.4 c.c. of a 24-hour Loeffler slant growth was the most satisfactory. A modified Neisser technic was reported by Zingher and Soletsky²⁶ in making intracutaneous virulence tests. They washed down a 24-hour Loeffler tube with 25 c.c. of sterile salt solution and injected 0.1 of

²² Weston and Kolmer, *Jour. Infect. Dis.*, 1911, 8, p. 295.

²³ *Jour. Am. Med. Assn.*, 1911, 56, p. 809.

²⁴ *Jour. Med. Research*, 1897-98, 2, p. 11.

²⁵ *Ibid.*, 1907-08, 17, p. 277.

²⁶ *Jour. Infect. Dis.*, 1915, 17, p. 454.

this intracutaneously. The advantage of their technic was that 4-6 different cultures might be tested on the same guinea-pig. A control animal was inoculated with the same amount of the suspension plus 0.5 c.c. of a 200-unit antitoxin serum.

Methods of Modifying Virulence.—Numerous attempts have been made in the past to modify the virulence of diphtheria bacilli. Unfortunately in many of these experiments no attempt has been made to distinguish between the toxigenic power and virulence. As has been shown in the case of diphtheria bacilli, there is an essential difference between organisms in this respect. Very early in the development of knowledge concerning the diphtheria bacilli, Roux and Yersin²⁷ and Funck²⁸ observed that diphtheria ran a more severe course when streptococci were present in large numbers, and experiments convinced them that in some way association in growth with streptococci caused an increased virulence in *B. diphtheriae*. Hilbert²⁹ found that this increased virulence was due to an increased ability to form toxin. The increased toxigenic power Escherich³⁰ ascribed to passage through susceptible animals, while others³¹ attributed the increase to the ability of the organism to proliferate more rapidly in a medium rendered more favorable by the growth of streptococci. Smirnow³² states that in his work streptococci had an antagonistic effect on diphtheria bacilli on blood serum during 10-15 hours' growth, but that after 20-30 hours this antagonistic action was not evident and might favor the growth. Another worker³³ grew two morphologically typical, but avirulent, cultures through 90 generations with virulent streptococci, transplanting every 3-4 days, and induced no increase in virulence.

More effort has been put forth in the attempt to increase virulence by animal passage. Trumpp³⁴ claims to have increased the virulence of an avirulent bacillus by passing it with toxin through guinea-pigs, to such a degree that a culture from the dead animal killed another guinea-pig. Hewlett and Knight³⁵ claim to have converted a pseudodiphtheria bacillus into a diphtheria bacillus by animal passage and heating, while Salter³ accomplished the same result by passage of the bacillus through canary birds. By cultivating the bacilli in collodion sacks in the peritoneum of the rabbit, Martin¹⁵ was able to increase the toxigenic power of diphtheria bacilli, including that of Park's No. 8, but not their virulence. It is claimed also by Ohlmacher² that the virulence of an avirulent strain was increased by passage through 1 guinea-pig. Unfortunately many of these results have not been confirmed, even tho carefully repeated by other investigators. The attitude of a majority of workers is well summed up by Kolmer, Woody, and Moshage,³⁶ who state: "The great weight of experimental evidence is to the effect that diphtheria bacilli which have proved nonvirulent with every test, remain so indefinitely despite prolonged efforts to give them even feeble pathogenic powers." It is thought more probable by many that the virulence of slightly pathogenic strains may be modified. It may well be that it is with such slightly pathogenic strains that

²⁷ Ann. de l'Inst. Pasteur, 1890, 4, p. 385.

²⁸ Ztschr. f. Hyg., u. Infektionskr., 1894, 17, p. 465.

²⁹ Ibid., 1898, 29, p. 159.

³⁰ Wien. med. Wchnschr., 1894, 11, p. 294.

³¹ Gibier, Compt. rend. Soc. de biol., 1897, 4, p. 392.

³² Jour. Med. Research, 1908, 18, p. 249.

³³ Williams, Jour. Med. Research, 1902, 8, p. 83.

³⁴ Centralbl. f. Bakteriöl., 1896, 20, p. 721.

³⁵ Tr. Brit. Inst. Prev. Med., 1897, i (1st series), p. 7.

³⁶ Am. Jour. Dis. Child., 1916, 11, p. 257.

early workers experimented. To quote the afore-mentioned authors again, "with these (strains with low pathogenicity) passage through animals or from throat to throat among men may readily enough restore a high degree of pathogenicity." In a long series of animal passages it may also be possible that another strain has been recovered at some stage, different from the one introduced, since members of this group are rather widely distributed.

A decreased virulence has been brought about by several methods. Artificial cultivation has apparently produced no lessened virulence in the case of Park's bacillus nor modified its toxigenic powers. It is stated in one case³⁷ that in 2 years' cultivation on artificial media the virulence was partially lost. Hewlett and Knight³⁵ report the reduction of virulence by heating for 17 hours at 45 C., but they were unable to repeat their results. In attempting to verify these findings, Williams³³ found no reduced virulence in diphtheria bacilli grown at 40-45 C., for several months. Roux and Yersin²⁷ found a slightly decreased virulence in dried membrane, while Abel³⁸ observed a similar quality in bacilli dried on silk threads for 86 days. A few instances of peculiar manifestations of low-grade virulence by diphtheria bacilli have been recorded. Abscesses were noticed by Cobbett³⁰ at the site of inoculation after the injection of nonvirulent organisms. From these abscesses he isolated diphtheria bacilli in purity. He reports having seen similar abscesses in guinea-pigs treated with large doses of virulent bacilli together with antitoxin and in an immunized horse treated with living bacilli. Councilman⁴⁰ reports an abscess around a hair follicle from which were isolated diphtheria bacilli that killed a guinea-pig in 48 hours. In a fatal case following typhoid fever a lung abscess contained *B. diphtheriae*. Heine¹⁷ more recently has suggested that his second group may possess pyogenic properties. One test⁴¹ to determine whether 'aggressins' such as Bail found were present in virulent diphtheria bacilli, gave negative results, as follows: Three guinea-pigs were injected with virulent diphtheria bacilli to obtain the aggressin. After death the peritoneal fluid was sterilized by filtration and injected with 2 avirulent strains. None of the animals was affected either with or without the aggressin.

While diphtheria is primarily a toxemic disease, a number of cases are on record in which an invasion of the blood stream has been observed. There has been little or no attempt to determine the relation of such penetration to the virulence of the organism. Mallory⁴² states: "In many fatal cases the bacilli get into the blood and can be obtained in cultures from various organs. The order of relative frequency is as follows: liver, kidneys, spleen, heart's blood and very rarely the brain. The highest percentage of septicemia recorded is fifty, but in this series general septicemia was found only in about 20%." ⁴² Another worker³⁸ twice isolated virulent diphtheria bacilli during life from the cerebrospinal fluid of a child suffering from general miliary tuberculosis and tuberculous meningitis. There were no clinical signs of diphtheria, but the organisms were present in the throat. One investigator⁴³ isolated diphtheria bacilli from the blood during life in 1 of 18 cases. In this case there was also a streptococcal infection, which he thinks may have predisposed to invasion. The bacteria were found only at one time, 9 days before death. Another

³⁷ Bardach, *Ann. de l'Inst. Pasteur*, 1895, 9, p. 40.

³⁸ *Deutsch. med. Wchnschr.*, 1895, 17, p. 545.

³⁹ *Jour. Hyg.*, 1901, 1, p. 497.

⁴⁰ *Jour. Med. Research*, 1896-97, 1, p. 14.

⁴¹ Zinsser, *Jour. Med. Research*, 1907-08, 17, p. 277.

⁴² Quoted by Nuttall and G. Smith, *The Bacteriology of Diphtheria*, 1908.

⁴³ Leede, *Ztschr. f. Hyg. u. Infektionskr.*, 1911, 69, p. 225.

worker⁴⁴ found diphtheria bacilli 3 times in 187 patients. Postmortem examination having given negative results, he concludes that bacteriemia, when it does occur, is temporary. Morgan⁴⁵ reports a case in which diphtheria bacilli found in the blood proved nonvirulent when tested on a guinea-pig. It is believed by some⁴⁶ that there is a temporary bacteriemia associated with every case of diphtheria at some time. One case in support of this theory is described by Wade,⁴⁷ a diphtheria bacteriemia in which 1600 bacilli to the cubic centimeter of blood were found to be highly virulent and to produce a toxin fatal in 0.015-c.c. amounts for guinea-pigs. In a number of cases reported, neither the virulence nor toxicity of the isolated culture had been tested, so that the results are without meaning in correlating the virulence with these factors.

In this study the methods adopted for determining the virulence and penetration of the 23 cultures tested were as follows: Sugar-free infusion broth with a reaction of ± 0.5 was inoculated with the pure culture and incubated at 37 C. for 48 hours. Unless a vigorous growth took place, several transfers were made in the broth until a good growth was obtained. Of this living unfiltered culture 0.2 c.c. was employed for the initial dose. After the total volume had been brought up to 4 c.c. with sterile salt solution, it was injected subcutaneously by means of a Hitchen syringe into guinea-pigs weighing 250-300 gm. If the animal died within 4 days, no further injections were made; if death did not occur in this time, 4 c.c. of a similar broth suspension were injected in the same manner into another guinea-pig. When death occurred, the animal was examined, and unless the typical postmortem findings for diphtheria were present, the death was not regarded as one due to diphtheria bacilli. The findings were typical of toxemia in all reported cases, consisting of marked induration and hyperemia at the site of injection, abundant gelatinous edema in the axillary and inguinal regions and often extending over the entire subcutaneous region of the belly, and enlarged hyperemic adrenals. Examination was made as soon after death as possible and cultures from the spleen, liver, and adrenals, and in many cases from the heart blood and kidney, were made to determine the power of penetration, with a view to correlating it with virulence. In taking the cultures, the surface of the organ was cauterized, a small incision made with sterile knife, and some of the pulp transferred to a tube of 0.5% dextrose broth with a reaction of ± 0.5 . These tubes were incubated at 37 C. for 4 or 5 days, and then transfers made to Loeffler's serum. After 24 hours' incubation the serum growth was stained with methylene blue for diphtheria

⁴⁴ Roedelius, *ibid.*, 1913, 15, p. 497.

⁴⁵ *Am. Jour. Dis. Child.*, 1913, 5, p. 317.

⁴⁶ Conradi and Bierast, *Deutsch. med. Wchnschr.*, 1912, 34, p. 1580.

⁴⁷ *Jour. Infect. Dis.*, 1915, 16, p. 292.

bacilli. (At first the organ pulp was transferred directly to Loeffler's serum also, but this method was not as satisfactory as the one described and was soon discontinued.)

The detailed results are given in Table 6. In all cases in which 4 c.c. were injected, 0.2 c.c. had proved harmless. It will be seen that of the 23 strains tested, 16 possessed high virulence, 4 low virulence, and 3 were avirulent. *B. diphtheriae* was isolated 8 times from 7 different animals. It was found 4 times in the spleen, and 2 times each in the liver and the adrenals. Five of the 7 animals received 2 c.c. of a strongly virulent strain, while the other two received a large dose of

TABLE 6
TESTS FOR VIRULENCE AND PENETRATION

Culture	Dose (c.c.)	Results	Recovery of Diphtheria Bacilli from Tissues				
			Spleen	Liver	Adrenal	Heart	Kidney
0	0.2	Died in 2 days	—	—	—	—	—
122	0.2	Died in 1½ days	—	—	—	—	—
905	0.2	Died in 2½ days	+	—	—	—	—
668	0.2	Died in 3 days	—	—	—	—	—
461	0.2	Died in 2¾ days	—	—	—	—	—
1290	4.0	Died in ¾ day	+	—	+	—	—
694	4.0	Lived	—	—	—	—	—
1303	0.2	Died in 1¼ days	+	—	—	—	—
1	0.2	Died in 1½ days	—	—	—	—	—
168	0.2	Died in 1¾ days	—	—	—	—	—
880	0.2	Died in 2¼ days	—	+	—	—	—
540	4.0	Died in ¾ day	—	—	+	—	—
42	0.2	Died in 3¾ days	—	+	—	—	—
600	4.0	Died in 1½ days	—	—	—	—	—
1293	0.2	Died in 1½ days	—	—	—	—	—
1441	0.2	Died in 1¾ days	—	—	—	—	—
Park's 8	4.0	Died in 2¾ days	—	—	—	—	—
49	4.0	Lived	—	—	—	—	—
50	4.0	Lived	—	—	—	—	—
2059	0.2	Died in 1¼ days	—	—	—	—	—
11	0.2	Died in 1¼ days	—	—	—	—	—
13	0.2	Died in 1½ days	—	—	—	—	—
20	0.2	Died in 2 days	..	—	—	—	—

organisms of rather low virulence. Since the 5 strains the organisms of which were recovered in the tissues showed no higher virulence than the 11 other strains, in which penetration was not found, it would seem that penetration is only a minor factor in the virulence of *B. diphtheriae*. The postmortem findings were typical of toxemia. It may be that bacteriemia, when present, is only temporary, and that blood examinations at intervals following injection, as suggested by Conradi and Bierast,⁴⁶ would give a larger proportion of positive results.

A series of experiments was conducted to determine whether the virulence of an avirulent strain could be increased either by animal passage alone, by symbiosis during passage with *Staphylococcus aureus*, or by injecting the culture together with sublethal doses of toxin into

successive guinea-pigs. For this purpose Strains 49 and 50 (described by Brown and Smith⁴⁸) were selected, because they were apparently true *B. diphtheriae* rather than *B. hofmanni*, it being deemed more probable that virulence would be increased in such strains than in the pseudodiphtheria bacilli. These strains were nontoxic, or nearly so, and avirulent. By Heine's classification, as may be seen by reference to Table 4, they belong to his first group, that should be virulent if the biometric test were taken as a guide, since both ferment dextrose, maltose, dextrin, and glycerin, but produce an alkaline reaction in saccharose. Both strains, when tested for virulence by the method described, produced no general or local effect. They were consequently considered nonvirulent.

In the first series each of the two strains was passed consecutively through 8 guinea-pigs weighing 250-300 gm., a 24-hour Loeffler culture being used for injection in each case. Four standard loops of the culture were added to 1 c.c. of sterile salt solution in a Hitchen syringe and the uniform suspension injected subcutaneously. After 48 hours the site of inoculation was sterilized with alcohol, a small incision made with sterile knife, and a platinum needle inserted, on which cultures were taken for transfer to Loeffler's slants. These were incubated, examined for purity, and another guinea-pig injected with the recovered culture. This series of tests was designed to show any increase in virulence due to passage alone and also to serve as a control for the other passages which were carried on simultaneously with *Staphylococcus aureus* and toxin. On the 7th passage, Strain 49 changed noticeably, from a long slender organism with few granules to a shorter highly granular organism. It showed no virulence when 4 c.c. of a 48-hour broth culture were injected subcutaneously into a guinea-pig. Strain 50 did not change in appearance with passage and was avirulent when injected in 4-c.c. amounts of a 48-hour broth culture. A change in morphology of these strains had been observed by Brown and Smith at different times during the 12 years the strains were in stock on artificial media, and the change is probably of no significance in this case.

In the second series of experiments the two atoxic strains, in association with *Staphylococcus aureus*, were passed through 8 guinea-pigs. Four standard loops of the diphtheria culture from a 24-hour Loeffler's slant were added to 1 c.c. of sterile salt solution in a Hitchen syringe with 2 loops of a 24-hour agar-slant culture of *Staphylococcus aureus*. This was thoroughly mixed and then injected subcutaneously into 250-

⁴⁸ Jour. Med. Research, 1914, 30, p. 443.

300-gm. guinea-pigs. After 48 hours a small incision was made aseptically at the site of inoculation and some of the edematous material taken with a looped needle and spread on Loeffler slants. After 24-48 hours the diphtheria colonies could usually be distinguished in some of the tubes and transferred to a sterile tube. This culture, after it had been examined for purity, was used for the next passage. The staphylococcus culture was always taken from the original culture and not from one passed through the series of guinea-pigs. By this method Strain 49 changed at the 5th passage, to a highly granular shorter form. It was not virulent when tested after the 8th passage in 4-c.c. amounts of a 48-hour broth culture. Strain 50 with *Staphylococcus aureus* did

TABLE 7
THE EFFECT OF ANIMAL PASSAGE AND SYMBIOSIS ON VIRULENCE

Treatment	Changed Morphology During Treatment	Virulence
Strain 49		
Before passage.....	After 7th passage After 5th passage	} Avirulent in 4-c.c. amounts
8 passages alone.....		
8 passages with <i>Staphylococcus aureus</i>		
8 passages with ½ M. L. D. toxin.....		
Strain 50		
Before passage.....		} Avirulent in 4-c.c. amounts
8 passages alone.....		
8 passages with <i>Staphylococcus aureus</i>		
7 passages with ½ M. L. D. toxin.....		
} Local abscess containing pure growth of diphtheria bacilli. No toxemia		

not change in morphology during passage. After the 8th passage the strain had acquired pyogenic properties; when 4 c.c. of a 48-hour broth culture were injected subcutaneously a nodule, the size of a hazelnut, formed at the site of injection. From this nodule, which contained purulent material, the diphtheria bacilli were recovered in pure growth after 4 days. No general symptoms of toxemia developed. This action is probably similar to that observed by Cobbett,³⁹ and Councilman,⁴⁰ and suggested by Heine as a property of his second group; it may indicate a low grade of virulence.

In the third series the two strains were passed through a series of guinea-pigs together with $\frac{1}{2}$ M.L.D. of diphtheria toxin. Strain 49 was passed through 8 guinea-pigs and Strain 50 through 7. Four loops

of a 24-hour Loeffler growth were injected subcutaneously, each time with $\frac{1}{2}$ M.L.D. of sterile toxin, and the diphtheria bacilli recovered after 48 hours from the site of injection. Strain 49 did not change morphologically during passage and was avirulent after 8 passages. Strain 50 became more granular and shorter after the 5th passage, and produced, after the 7th passage, a local abscess at the site of injection from which a pure growth of diphtheria bacilli was isolated. No general toxic effects were observed.

The results of these experiments, under the conditions established, may be summed up as follows: (1) passage through susceptible animals did not increase virulence even in strains whose biometric reactions are typically those of true diphtheria; (2) passage with *Staphylococcus aureus* may have caused the acquisition of a low grade of virulence in some cultures; (3) passage with toxin may also have caused the acquisition of a low grade of virulence in some cases.

It was thought worth while to determine the number of organisms that are needed to produce fatal results within 4 days, and to demonstrate the relation that this might bear to virulence. The difficulties encountered in accurately determining the number of bacilli injected have been manifold. Four methods have been tested for counting the organisms injected:

(1) The standard platinum-loop method. It was very soon found that the dry growth of diphtheria bacilli on Loeffler's serum could be measured with no degree of accuracy with the platinum loop.

(2) Wright's method for standardizing vaccines was employed—washing down the growth on a Loeffler's tube, breaking up the clumps with sterile glass beads in a test tube, and then counting the organisms stained with methylene blue diluted 5 times and comparing the result with the number of erythrocytes. In all cases 500 organisms were counted from two slides. This method gives reasonable satisfaction, tho there are certain objections to its use; living bacteria cannot be distinguished from dead, and there is often great difficulty in obtaining a uniform distribution of blood and organisms, resulting in an unintentional selection of fields to be counted in an effort to find thin fields.

(3) One-percent-glycerin agar plates were made of the diluted suspension and the bacteria counted after 48 hours' incubation at 37 C. This method has the advantage of showing only living organisms, but it may be questioned whether all living organisms will grow sufficiently in glycerin agar to be counted at 48 hours, for, as is well known, diphtheria bacilli do not grow vigorously on agar and strains differ in their ability to grow on this medium.

(4) The organisms were washed down in sterile salt solution, shaken thoroughly with glass beads, and the organisms counted with a Thoma hemacytometer. Methylene blue diluted 5 times was used as a diluent and stain, and the organisms were counted with a Leitz No. 7 objective and No. 4 ocular. This method is open to the same objection as Wright's method in not distinguishing between living and dead organisms. Altogether, however, it

was considered the most satisfactory of the methods employed. Any method of counting diphtheria organisms is rendered difficult by the well-known tendency of the organisms to clump. This difficulty was eliminated as much as possible by shaking thoroughly with glass beads. The objection that the 2nd and 4th methods do not distinguish between living and dead organisms has been met by using only 24-hour cultures, in which a maximal number of organisms are viable. It is not contended that for any culture tested the three methods give identical results, because it has been found that there may be high percentages of difference.

The minimal fatal doses of living organisms of 5 different strains were carefully determined by injecting subcutaneously into standard guinea-pigs known numbers of organisms, obtained by the plating and blood-counter methods. These results are compared in Table 8 with

TABLE 8
RELATION OF THE NUMBER OF ORGANISMS INJECTED TO VIRULENCE

Culture	Virulence (shown by injection of 48-hour broth culture)	Organisms Necessary for Fatal Results			
		Fraction of 24-hour Loeffler Slant	Number by Blood Counter	Fraction of 24-hour Loeffler Slant	Number by Plating
Park's 8	Low	1.0	1,930,000,000	2.4	152,000,000*
2059	High	0.0004	6,592,000	0.01	19,900,000
11	High	0.0001	1,216,000	0.001	1,210,000
13	High	0.001	1,520,000	0.001	780,000
20	High	0.02	160,000,000	0.005	15,650,000

* Not fatal.

the results for the same strains determined by the routine method described earlier in this paper. It will be seen that the number of organisms necessary to produce fatal results was much greater for a strain of low virulence, such as Park's No. 8, than for the other four highly virulent strains. In one case the growth on 2.4 Loeffler slants washed down in salt solution did not produce fatal results, but subsequently the same strain from another laboratory produced fatal results when the growth from one tube was injected. A 4-c.c. dose of a 48-hour broth culture of this organism was uniformly fatal. Inasmuch as such variations do occur when washed cultures are injected, it would seem advisable to adopt uniform methods of testing for virulence in public health laboratories. Undoubtedly, the virulence in this case when tested with the broth culture was due to the toxin contained in the broth. This culture, however, might have been classed as avirulent had the washed-down growth alone been used.

Experiments were performed after the methods of Rosenow¹⁰ to ascertain whether there were virulins present in the highly virulent

strains that could increase the virulence of low-virulent strains or render sublethal doses of virulent organisms lethal.

Strain 11 was selected for the production of virulins because of its high virulence. The organisms were grown on the surface of 15 Loeffler tubes for 24 hours, and then were washed down in a total volume of 50 c.c. $\frac{M}{8}$ NaCl. This emulsion was kept in the incubator for 48 hours at 37 C. and then was filtered through a Berkefeld filter and tested for sterility. This constituted Virulin A. Virulin B was prepared by washing down 15, 24-hour Loeffler tubes with 50 c.c. of sterile water. This was kept in the incubator for 6 hours and then rapidly frozen and thawed 4 times. The suspension was then centrifuged and the clear supernatant fluid filtered through a Berkefeld filter. The strains used with the virulins were Park's No. 8, and Strains 13, 11, and 20. These were grown on Loeffler slants and an emulsion made in sterile salt solution. Sublethal doses, or doses approximating the lethal, were injected together with the virulin.

Table 9 shows that no increase in virulence was induced in any case except in Strain 20 with Virulin A. The guinea-pig in this case died on the 10th day, while the control animal lived. In 2 cases the virulin seemed to have some protective action. In general, it may be said that the virulin did not influence the virulence to any degree.

The Bail method of obtaining aggressins was also employed.

TABLE 9
'VIRULINS' AND THEIR EFFECT ON VIRULENCE

Strain	Proportion of Loeffler Slant in 1 c.c. NaCl	Amount of Virulin (c.c.)	Results
Control	0	3 Virulin A	Lived. No local action
Park's 8	0.2	3 Virulin A	Lived. Marked necrosis and edema
Park's 8	0.2	0 (3 NaCl)	Lived. Marked necrosis and edema
13	0.0001	3 Virulin A	No local action
13	0.0001	0 (3 NaCl)	No local action
11	0.0001	3 Virulin A	Died in 10 days. Severe edema
11	0.0001	0 (3 NaCl)	Died in 4 days. Edema and necrosis
20	0.0002	3 Virulin A	Died in 10 days. Edema and necrosis
20	0.0002	0 (3 NaCl)	Lived. Edema and necrosis
Control	0.00	3 Virulin B	No local action
Park's 8	0.2	3 Virulin B	Lived. Edema and necrosis
Park's 8	0.2	0 (3 NaCl)	Lived. Edema and necrosis
13	0.0001	3 Virulin B	Lived. Edema and necrosis
13	0.0001	0 (3 NaCl)	Died in 3 days. Animal accidentally destroyed without autopsy

Rabbits were first used in hopes of obtaining a large amount of peritoneal exudate. One cubic centimeter of a 48-hour culture on sugar-free broth of a highly virulent strain, No. 2059, was injected intraperitoneally into each of 2 rabbits and the same amount of another virulent strain, No. 11, was injected into each of 2 other rabbits. These rabbits were bled to death after 4 days and the peritoneal exudate taken. Very little exudate had been formed, altho there was a membrane present on the viscera of all the rabbits. A total of 5 c.c. was collected. The blood was defibrinated as drawn and pooled from the

4 rabbits for the tests. The corpuscles were thrown down by centrifugation, as well as the cellular material in the peritoneal exudate. The organisms in the exudate were killed by 5% phenol, the fluid was tested for sterility by plating, and tests were made with the exudates and serum as outlined in Table 10. With the small amount of material it was impossible to make tests

TABLE 10
'AGGRESSINS' AND THEIR INFLUENCE ON *B. DIPHTHERIAE*

Culture	Proportion of Loeffler Slant in 1 c.c. Fluid	Amount of Aggressins (c.c.)	Amount of NaCl Solution (c.c.)	Result
49 (passed with <i>Staphylococcus aureus</i> through 8 guinea-pigs)	0.33	3 (rabbit blood serum)	0	No effect
Serum control.....	0	3 (rabbit blood serum)	1	
49 (passed with <i>Staphylococcus aureus</i> through 8 guinea-pigs) control	0.33	0	2	
49 (passed with <i>Staphylococcus aureus</i> through 8 guinea-pigs)	0.33	2 (rabbit peritoneal fluid)	1	
Rabbit-peritoneal-fluid control	0	2 (rabbit peritoneal fluid)	2	
50 (passed with toxin through 6 guinea-pigs)	0.33	3 (guinea-pig peritoneal fluid)	0	
50 (passed with toxin through 6 guinea-pigs) control	0.33	0	3	
Guinea-pig-peritoneal-fluid control	0	3 (guinea-pig peritoneal fluid)	1	
Park's No. 8.....	0.2	3 (guinea-pig peritoneal fluid)	0	
Park's No. 8 control.....	0.2	0	3	
49 (passed with <i>Staphylococcus aureus</i> through 8 guinea-pigs)	0.33	3 (guinea-pig peritoneal fluid)	0	No effect
49 (passed with <i>Staphylococcus aureus</i> through 8 guinea-pigs) control	0.33	0	3	

with controls on more than 1 strain. Strain 49, which in conjunction with *Staphylococcus aureus* had been passed through 8 guinea-pigs, was used. This strain had changed in morphology during passage, but not in virulence. It will be seen that the exudate caused no increased virulence in this case.

Since the peritoneal exudate in rabbits was so slight, guinea-pigs were substituted. Six large guinea-pigs were injected intraperitoneally with the same strains, Nos. 2059 and 11, 1 c.c. of a 48-hour broth culture being used in each

case. One animal injected with each strain died before the expiration of 4 days; the rest were killed at that time and the peritoneal exudate taken. The amount of exudate varied in the different guinea-pigs from 2 to 15 c.c., but all showed some lesions and membrane. After all the fluid possible had been obtained, the viscera and peritoneal cavity of each guinea-pig were washed with 10 c.c. of salt solution and the washing added to the exudate. The combined fluid was centrifugated and the supernatant fluid sterilized by passing through a Berkefeld filter. Three strains were used to test the potency of the filtrate—Strain 50 after 6 passages through guinea-pigs with toxin, Park's No. 8, and Strain 49, which together with the staphylococcus had been passed through 8 guinea-pigs.

The results are recorded in Table 10. In no case did the aggrassin increase the virulence.

THE RELATION BETWEEN BIOMETRIC REACTIONS AND VIRULENCE

The fermentation reactions of diphtheria bacilli have been considered one of the most satisfactory bases of classification within the diphtheria group. There is little question but that true diphtheria bacilli ferment dextrose much more vigorously than do the pseudo-diphtheria bacilli, and that strong dextrose-fermenters are more likely

TABLE 11
RELATION BETWEEN BIOMETRIC REACTION AND VIRULENCE

Carbohydrate	Reaction	Virulence				
		High	Medium	Low	Avirulent	Not Tested
Dextrose.....	{ 2 below 3.5.....	1	0	0	0	1
	{ 19 from 3.5 to 4.5....	12	1	2	2	2
	{ 5 over 4.5.....	3	0	1	1	0
	{ 15 acid.....	8	1	2	3	1
Maltose.....	{ 2 neutral.....	2	0	0	0	0
	{ 9 alkaline.....	6	0	1	0	2
	{ 3 acid.....	2	1	0	0	0
Saccharose.....	{ 2 neutral.....	1	0	0	1	0
	{ 21 alkaline.....	13	0	3	2	3
	{ 10 acid.....	8	0	0	2	0
Dextrin.....	{ 3 neutral.....	2	0	0	0	1
	{ 13 alkaline.....	6	1	3	1	2
	{ 20, 2 or below.....	13	1	2	1	3
Glycerin.....	6 over 2.....	3	0	1	2	0

to be virulent. No test other than a virulence test has as yet been found by which true virulent diphtheria bacilli can be differentiated from the diphtheria bacilli that are avirulent. Heine¹⁷ has sought to distinguish such cultures by their fermentation results on different sugars. His 1st group of virulent organisms ferments dextrose.

maltose, lactose, and dextrin, but not saccharose. Lactose, which forms one of his important differential sugars, was not used with the 26 strains that were tested in my experiments. That being left out of consideration, 10 of the 26 strains belonged in this group; 7 of these were highly virulent, 2 were avirulent, and 1 was not tested. Three of the other strains probably belonged to Heine's 2nd and 3rd groups; of these, 2 were highly virulent and 1 was of medium virulence. A summary of the relation between virulence and fermentation on 5 carbohydrates is given in Table 11. From this table it will be seen that there is no constant relation between these two characteristics in dextrose, maltose, saccharose, dextrin, and glycerin. The greater number of highly virulent strains produce between 3.5 and 4.5% of normal acid in dextrose, give acidity in maltose and dextrin, and less than 2% acid in glycerin, with alkalinity in saccharose. In none of these carbohydrates is there a constant production of definite acidity or alkalinity among highly virulent strains. None of the strains that were of low virulence or avirulent, however, produced any acid in saccharose, altho 2 virulent strains fermented this sugar.

TOXIN-PRODUCTION IN RELATION TO VIRULENCE

The factors that enter into the production of toxin are as complex and varied as those that enter into virulence. Indeed, many writers have made no distinction between toxicity and virulence, using the terms synonymously. Nuttall and Graham Smith¹⁸ state: "It cannot be said that there is any very marked correspondence between the severity of the disease in the human subject and the virulence or toxigenic power of the bacillus obtained from the case." Moshage and Kolmer¹ believe that acid-production may run parallel to virulence. Neisser and Ginns⁴⁹ state that often diphtheria bacilli that are extremely toxic to the guinea-pig may be rather mildly virulent for man. Sometimes also cultures virulent for man are only mildly toxic to the guinea-pig. Such statements naturally bring up the question as to what relation may exist between toxicity and virulence.

It is conceivable that virulent strains which produce a feeble toxin *in vitro* may be able to produce a strong toxin *in vivo* that may be the sole factor in virulence, for it is well known that slight changes in the broth composition profoundly affect the amount of toxin produced. It does seem, however, that these factors whatever they might be.

⁴⁹ Kolle and Wassermann, *Handb. d. pathogen. Microorganismen*, 1913, 5, p. 962.

would be operative alike in different strains; for example, two equally virulent strains would be expected to produce toxin *in vitro* in the same broth under identical conditions in about the same degree, if toxin alone were responsible for their virulence. Some have thought that in certain strains the toxin may be contained largely in the bodies of the bacilli. It has been reported, however, that the bodies of the diphtheria bacilli do not at any time contain toxin in any considerable amounts.⁵⁰ This is also indicated by my virulin experiments, in which the extracted fluid seemed to be entirely without toxic effects.

The demand for diphtheria antitoxin has resulted in much fruitful work in the production of toxins. Hida⁵¹ called attention to the importance of the kind of peptone used, having found that a peptone containing deuterio-albumose in considerable amounts gives the most potent toxins. Park and Williams⁵⁰ found, as have others since, that 2% of peptone gives the best results. The importance of good peptone in toxin-production has been further emphasized since the supply of Witte peptone was cut off by the war. I found, as did others, that the ability of a strain to produce a potent toxin was considerably lower when certain American-made peptones were used in the medium than when the Witte peptone was employed. The product of the autodigestion of hog stomach has given very good results when used as a substitute for peptone. The kind of meat used for the infusion broth has in many cases had an effect on the strength of the toxin. Usually beef infusion or veal broth is used, many preferring the latter for obtaining a high toxin. It has been found that proteins were not essential to toxin-formation, that a potent toxin could be formed on nonprotein synthetic media, one which would kill the guinea-pig in 36-38 hours.⁵² Other conditions, as the amount of dextrose, have been emphasized by Spronck⁵³ as of prime importance in toxin-production. The relation of oxygen and the production of a heavy surface growth have been discussed by different writers. Undoubtedly all of these factors do play a part in the production of toxin. The production of a heavy surface film does not necessarily indicate a strong toxin, altho a strong toxin is rarely formed unless a good surface growth occurs. Park and Williams⁵⁰ state that in their experiments, when other conditions were similar, the strength of the toxin was in proportion to the virulence and vigor of the growth of bacilli employed.

Strains of diphtheria bacilli isolated from clinical cases vary widely in their ability to produce toxin. It is not difficult to get toxins with an M. L. D. of 0.1, 0.05, or even 0.03 c.c. from such sources, but strains producing a toxin with an M. L. D. of less than 0.01 c.c. are very rare. The Park strain has, however, continued to give a very strong toxin, 0.007-0.001 for two decades. Fruitless efforts have been made by Smith and Walker⁵⁴ and Berry and Blackburn⁵⁵ to find a strain producing a stronger toxin than this. Of the 46 cultures tested by Smith and Walker, 12 gave an M. L. D. between 0.036 and 0.06, 21 between

⁵⁰ Park and Williams, *Jour. Exper. Med.*, 1896, 1, p. 164.

⁵¹ *Ztschr. f. Hyg. u. Infektionskr.*, 1908, 61, p. 273.

⁵² Hadley, *Jour. Infect. Dis.*, 1907, Suppl. 3, p. 95. Ushinsky, *Centralbl. f. Bakteriöl.*, 1897, 21, p. 146.

⁵³ *Ann. de l'Inst. Pasteur*, 1895, 9, p. 758.

⁵⁴ *Jour. Med. Research*, 1897-98, 2, p. 12.

⁵⁵ *Jour. Infect. Dis.*, 1912, 10, p. 404.

0.07 and 0.09, and 9 between 0.10 and 0.12. Of the 100 cultures tested by Berry and Blackburn, 9 did not produce fatal results when 3 c.c. were injected, 27 had an M. L. D. of 1 c.c., 11 of 0.05, 6 of 0.033, 25 of 0.02, 11 of 0.01, and 3 of 0.005. It is pointed out that the Park strain grown under the same conditions has varied in the toxin produced from an M. L. D. of 0.05, to 0.003, 0.0015, and 0.0005 c.c.⁶⁶ Indeed, it is rare to find 2 flasks growing under identical conditions that give the same M. L. D., altho the variation may be slight.

In order to correlate virulence, toxin-production, and acid-production, toxin tests were made on a number of different strains some of which were avirulent, some of low virulence, and some of high virulence. The production of toxin at the time this study was undertaken was complicated by the use of an American peptone which was of little value in producing toxin. Park's strain, and Strains 20, 11, and 2059 did not produce enough toxin with this peptone to kill a guinea-pig in repeated tests with 1-c.c. amounts, while of Strain 11, 0.7 c.c. was required to produce fatal results. Later, when Witte peptone was substituted, strong toxins were obtained from these same strains.

The methods of producing toxins were as follows: The medium used was beef infusion broth prepared in the usual way, and rendered sugar-free. This was titrated carefully to +1.2, and sterilized by the discontinuous method in 250-c.c. Erlenmeyer flasks containing 75 c.c. of the medium. Sufficient 10% sterile dextrose solution was then added to each flask to make a dextrose content of 0.2%. Some of the same medium was filled into test tubes and the organisms trained to grow on the surface before transference to the flasks. After the surface growth was transferred to the flasks, the latter were incubated at 35-36 C. for 10 days. The flasks were then examined for purity of growth and if pure, 0.5% phenol was added and the flasks placed in a dark ice box over night. The material was filtered the next morning through filter paper and then through a Chamberland filter, and tested for purity. Initial tests were made by injecting subcutaneously 1, 0.7, 0.3, and 0.1 c.c. of the toxin into 250-300-gm. guinea-pigs. All animals that died were examined and are included only if there was typical toxemia.

Table 12 gives the results of these tests, together with the virulence and biometric results for the same strains. The strongly virulent strains all produced strong toxins, strains with low virulence in all cases produced fatal toxins, while avirulent strains in all cases failed to produce any toxins that were lethal in large doses. One low-virulence strain, Park's No. 8, produced the most potent toxin, while the other three of low virulence were not such strong toxin-producers as three of the high-virulence strains. Of the toxin-producers, all fermented dextrose and glycerin, 7 fermented maltose, 2 saccharose,

⁶⁶ Kolle and Wassermann, *Handb. d. pathogen. Microorganismen*, 1913, 5, p. 965.

and 5 dextrin; of the non-toxin-producers, all produced acid in dextrose, maltose, dextrin, and glycerin, and none fermented saccharose. The number of strains tested is too few to permit of any far-reaching conclusions, but it does not seem that the acid-production in the five sugars can be taken as an index of toxin-production.

TABLE 12
TOXIN-PRODUCTION AND VIRULENCE

Culture	Biometric Reactions					Virulence	Toxin M. L. D.
	Dextrose	Maltose	Saccharose	Dextrin	Glycerin		
20	+	+	—	+	+	High	0.05
13	+	+	—	+	+	High	0.1
11	+	+	+	+	+	High	0.0075
2059	+	+	—	+	+	High	0.5
Park's S	+	—	—	+	+	Low	0.0075
540	+	—	—	—	+	Low	0.7
600	+	+	—	—	+	Low	0.3
1290	+	+	+	—	+	Low	0.3
694	+	+	0	—	+	Avirulent*	1.0 No action
50 (passed 6 times with toxin)	+	+	—	+	+	Very low.* Abscess- formation	4.0 No action

* Not fatal. + = acid; — = alkaline.

The relationship between the number of organisms that are necessary to produce fatal results and the toxin-production is seen in Table 13. Five strains only are compared. The virulence and the number of organisms necessary to produce fatal results run in nearly reverse order. There is apparently no relationship between the minimal fatal

TABLE 13
RELATIONSHIP BETWEEN THE NUMBER OF ORGANISMS INJECTED, TOXIN-PRODUCTION, AND VIRULENCE

Strain	Number of Organisms Necessary for Death	Toxin M. L. D.	Virulence 48-hour Broth Culture
Park's No. S	1,930,000,000	0.0075	Low
20	160,000,000	0.025	High
13	1,520,000	0.05	High
11	1,216,000	0.0075	High
2059	6,592,000	0.5	High

dose of live organisms and the M.L.D. of toxin, for the Park strain is the strongest toxin-producer, yet a larger number of organisms are required to produce fatal results. Strain 2059 has relatively weak toxigenic powers, yet fewer bacteria are necessary for fatal results than in the case of Strain 20, which has double the toxigenic powers.

SUMMARY AND CONCLUSIONS

There is a maximal acid-production for each strain in various carbohydrates. The greater the relative exposure to oxygen of the air, the more rapid is the production of acid.

The time of the maximal production of acid in the various carbohydrates varies not only with the carbohydrate used, but also with different strains. A uniform time, 8 days, for reading the final results in all carbohydrates will give the characteristic biometric results. The lack of uniformity in the reported results of various workers may be due to the use of different indicators and to reading the results at different periods of incubation.

A dextrose, maltose, saccharose, or dextrin content of 0.5% gives as satisfactory biometric results as of 1%.

The biometric results on 26 strains of morphologically true diphtheria show that all fermented dextrose and glycerin, the majority (23) produced no acid in saccharose, 15 fermented maltose, and 13 dextrin.

Twenty-three strains were tested for virulence and the power of penetration in order to determine whether penetration and virulence are related. Sixteen strains were highly virulent, 4 possessed low virulence, and 3 were avirulent. Bacilli were recovered from the tissues 8 times. The virulence of the culture seemed to be of less importance in relation to penetration than the number of organisms injected.

No increase in virulence was produced in two morphologically and biometrically typical atoxic strains by passage through 8 successive guinea-pigs.

When the same avirulent strains were passed, together with *Staphylococcus aureus*, through 8 successive guinea-pigs, one of the two acquired slight pyogenic powers for the guinea-pig, but produced no general effect or toxemia. The other strain was unchanged.

Passage of the same avirulent strains together with $\frac{1}{2}$ M.L.D. of toxin through 7 and 8 successive guinea-pigs produced pyogenic powers in the same strain that was modified by passage with *staphylococcus*. This may indicate a very low grade of virulence.

The use of the Thoma hemacytometer gives the most satisfactory results in counting the number of diphtheria bacilli injected. The bacilli are well stained for counting in Loeffler's methylene blue diluted 5 times.

The minimal fatal dose of living diphtheria bacilli seems to be greater in strains of low virulence.

Some strains of bacilli may be classed as avirulent when tested by using the washed-down growth from a Loeffler tube that would be considered virulent if tested by injection of a 48-hour broth. This difference is undoubtedly due to toxin formed in the broth.

'Virulins' prepared by a method similar to that employed by Rosenow did not modify the virulence when injected with sublethal doses of virulent organisms.

In no case did 'aggressins' prepared by the Bail method increase the virulence or produce fatal results when injected with sublethal doses of virulent strains.

No constant relationship exists between virulence and the fermentation reactions in dextrose, maltose, saccharose, dextrin, and glycerin. All virulent strains (and many avirulent) ferment dextrose and glycerin, usually give an acid reaction in maltose and dextrin, but do not ferment saccharose. Exceptions occur, however, in the fermentative action of virulent strains on maltose, dextrin, and saccharose.

The biometric reactions cannot be taken as indicative of toxin-production. They do not run parallel.

Toxin-production and virulence do not run parallel in many cases. The number of organisms necessary for fatal results is a good indication of the virulence, but gives no idea of the toxicity of the strain.

GERMICIDAL ACTION AND SPECIFIC BACTERIOTROPISM
OF QUININ AND UREA HYDROCHLORID, ETHYL-
HYDROCUPREIN, AND OTHER CINCHONA
DERIVATIVES ON PNEUMOCOCCI
IN VITRO *

STUDIES IN PNEUMONIA. I

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It has been long and generally known that quinin possesses certain antiseptic and bactericidal properties, and there is a long-standing tradition, endorsed by such clinicians of an elder day as DaCosta¹ and Bartholow,² that quinin given sufficiently early and in sufficiently large dose, will arrest an attack of pneumonia. Many physicians have advocated — as others with equal earnestness have opposed — the use of quinin salts as a routine treatment in this malady.† Among the former, Juergensen,³ Schultz,⁴ Aufrecht and Petzold,⁵ Henry,⁶ Gibson,⁷ and Galbraith⁸ may especially be cited. One of us (S. S. C.)⁹ has for many years — since 1904 systematically, as part of a definite plan for the

† Since this clinical testimony is at present largely ignored or forgotten, a few illustrative quotations from teachers of international repute, as well as from a country practitioner of large experience, are worth making note of.

"Administered at the critical moment," says Bartholow (1876), "a commencing fibrinous pneumonia . . . may be suppressed by a full dose (20 to 40 grains). Its power in this respect is much increased by combination with morphia. If the time has passed for the use of quinia in this way, it is employed with advantage . . . in . . . small doses (2 to 4 grains) . . . given frequently."

One of us (S. S.-C.) repeatedly heard DaCosta state in the course of that master's lectures on the practice of medicine (about 1880) that full doses of quinin administered shortly after the initial chill, will often arrest the development of acute lobar pneumonia. "It is difficult to prove this fact," DaCosta

* Received for publication November 27, 1916.

¹ From a student's notes of lectures (1880 to 1883).

² *Materia Medica and Therapeutics*, 1878, p. 141.

³ Von Ziemssen's *Cyclopedia of the Practice of Medicine* (Eng. translation), 1875, p. 165.

⁴ *Jour. Am. Med. Assn.*, 1886, 7, p. 119.

⁵ *Deutsch. Arch. f. klin. Med.*, 1901, 70, p. 373.

⁶ *Tr. Assn. Am. Phys.*, 1911, 26, p. 175.

⁷ *Glasgow Med. Jour.*, 1911, 75, p. 321.

⁸ *Jour. Am. Med. Assn.*, 1904, 43, pp. 108, 608.

⁹ *Tr. Assn. Am. Phys.*, 1911, 26, p. 169. *Am. Jour. Med. Sc.*, 1912, 143, p. 40.

treatment of pneumonia — employed large doses of the double hydrochlorid of quinin and urea by subcutaneous and intramuscular injection and, more recently, by intravenous injection; and has made a number of reports of its apparently favorable influence.¹⁰

The research of which the present communication reports but a single preliminary phase, is the long delayed fruition of his hope to put to experimental test certain opinions formed and expressed as the result of the therapeutic experience cited. Altho the friend and patient whose generosity has made it possible to begin the work declines to have his name attached to it, that generous assistance may nevertheless be acknowledged thus impersonally.

The validity of the clinical observations cited being assumed, we

would go on to say, "because one has no means of showing that what is absent was on the way. Yet it is true. The fact that pneumonia sometimes aborts spontaneously, or runs a very short course, does not negative this view. The real difficulty is that one rarely sees his patient soon enough."

Perhaps the most outspoken of modern champions of the use of quinin in pneumonia is Juergensen (1871), who writes:

"For children I use a grain and a half (0.1 Gm.) for every year up to five years of age, and after that period from seven to fifteen grains (0.5 to 1 Gm) according to circumstances. These quantities may be exceeded without doing any harm. When the fever is intense, seventy-seven grains (5 Gm.) may be given to a strong adult, and fifteen grains (1 Gm.) to a child under one year, always in one dose. I have repeatedly used both these amounts. I have acquired my experience by gradually increasing my doses, and I have never seen any harm done; in fact it is my firm belief that even these are not the extreme limits as to quantity. . . . I know that many will be alarmed at such large doses. My teacher is experience. Only fools resist facts. . . . The heart is the guide. . . . Whoever carefully examines the pulse walks safely; whoever fails to do it, easily stumbles. I have never noticed any harm done by quinin. The pulse falls at the same time with the temperature, but remains full and strong, or is improved in character if it had previously been weak. The discomfort on the part of the patient is generally not much greater than from small doses, and disappears rapidly."

O. T. Schultz of Mt. Vernon, Ind., "a small country town in the confluence of the Ohio and Wabash Rivers," states that his "practice largely lies in the 'bottoms' of these rivers, and among farmers more or less exposed to the vicissitudes of a country that is being opened up and brought under cultivation." From May, 1875, to May, 1886, he had under observation 238 cases of croupous pneumonia, not malarial, "few of which presented the sthenic type. Under treatment with quinin, supplemented by zinc phosphide, and by camphor and digitalis, when necessary, the mortality was 6.5 per cent."

He explains that he means by typhoid pneumonia, "a croupous pneumonia that in an enfeebled constitution runs an adynamic course," and he makes the following acute comment:

"How quinia accomplishes good results . . . has not as yet been deter-

¹⁰ Med. News, 1892, 61, p. 216. Pennsylvania Med. Jour., 1900, 9, p. 426. Internat. Clin., 1912, 3, p. 56. Jour. Am. Med. Assn., 1913, 61, p. 107.

wished to determine, if possible, (1) how quinin exerts its favorable influence on the acute pneumonias of man, and (2) how this influence, as a whole or in any of its various phases, may be reinforced or made more definite.

The first of these questions leads to an investigation (1) of the germicidal powers of the quinin compounds and other cinchona derivatives; (2) of their power to modify in any way the aggressive action of the infective agent; (3) of their power to modify in any way the results of infective action, including the development and effects of any or all of the poisons taking part in the toxic complex, and (4) of their effects, direct and indirect, by way of excitation or by way of supplementation, of the various defensive processes (leukocytosis, phago-

mined. I opine that it is not by its fever-lowering property, for I have seen the general condition . . . improved even when no impression was made on the temperature. . . . Our typhoid pneumonia is very *fatal* and quinia given in large—antipyretic—doses may not reduce the morning temperature at all, and yet . . . influence for good the course of the disease. There seems to be inherent in quinia, when given in large doses, a specific power of so influencing diseases attended with great and rapid tissue metamorphosis and retention of the waste-products of such tissue changes in the system, that these diseases take on a more favorable course and come to a more happy issue under its use."

F. P. Henry, in discussing the report made by one of us in 1911 to the Association of American Physicians stated that, following Aufrecht and Petzold, he had resorted to the subcutaneous use of quinin salts continuously in his cases of pneumonia since early in 1902. "I have never published a report of my cases in detail," he added, "but in a paper . . . in the *Philadelphia Medical Journal*, February, 1903, I referred to this method of treatment, mentioned my employment of it, and heartily endorsed it. I corroborate all that Dr. Cohen says in its favor. I have not used the large doses which he employed. . . . They did not seem to me to be necessary, and this may be because the preparation which I employ . . . the hydrochlorosulphate . . . is richer in quinin than the double salt of quinin and urea."

G. A. Gibson of Edinburgh writes: "In cases showing severe toxemia and great leukocytosis, much may be done by the employment of quinin. In several instances of this kind—in which the patients had even reached a stage of profound coma, with complete relaxation of the sphincters, and every evidence of imminent danger—the hypodermic use of quinin has produced the most remarkable effect. The best preparation for this purpose is the acid hydrochloride, which is extremely soluble, and which may be administered hypodermically in doses of 2 grains every two hours, or every hour. No more gratifying results have ever occurred in my own hands than from this method of treatment."

And a few months before his death, in a letter to one of us, he wrote that by the latter's reports he had been encouraged to use quinin more boldly, especially when it was guarded by cocain or a posterior-pituitary preparation as recommended, and that he believed that he had saved the life of "a dear friend 80 years old" in that way.

cytosis, neutralization of toxic products, and the like), and specifically among these the conversion of termination from crisis into lysis; this involving, in turn, an investigation into the causes and mechanisms of natural crisis. It requires also (5) an investigation of the toxic properties of the drugs studied.

The second of these questions leads into the field which Morgenroth¹¹ has begun to explore; namely, chemical modification of the drug molecule. Morgenroth's drug, optochin (ethylhydrocuprein), a derivative of hydroquinin* (methylhydrocuprein), has been shown by him and others to be immeasurably superior as a pneumococcide in vitro and in vivo to any quinin compound. This work constitutes the first successful attempt toward evolving a chemotherapeutic agent for a bacterial infection, and as such, should be emphasized. However, as will appear in the course of our various reports, optochin does not exhibit the same high degree of superiority throughout the whole range of actions studied, as it does when considered from the viewpoint of bactericidal activity alone; nor do clinical reports of its use in the acute pneumonias of man show it to be greatly, if at all, superior to the ordinary salts of quinin, even when reinforced by antipneumococcic serum.† Moreover, its greater toxicity renders it less available than the more commonly used cinchonics, as a therapeutic agent in the hands of the general practitioner under the common conditions of practice. Hence, since the therapeutic problem in pneumonia is only in part one of a bactericide, and the other properties of quinin are therefore to be studied and if possible, reinforced—with a lowering rather than an elevation of toxicity—there is still much to engage the attention of workers in this special field.

This report is confined to certain preliminary studies concerning the germicidal action of quinin, its compounds, and its congeners, especially their action on pneumococci. Altho a like work had been done, in part, by previous observers, yet since the research contemplated also certain

* Hydroquinin is found in the cinchona bark, but the commercial preparation is made synthetically. Optochin is synthetic only (see Paper 3 of this series).

† Moore¹¹ has shown that in experimental pneumococcal infection the conjoined use of an appropriate immune serum (according to the type of infection) and ethylhydrocuprein, affords a much greater degree of protection than the use of either alone. Lenne,¹² however, whose clinical work was based on experimental observations by Engwer and by Boehnke similar to those of Moore, did not get equally favorable results from the conjoined treatment. Using the polyvalent serum of Neufeld-Handel, he found the mortality in small groups of cases to be 11.8% with ethylhydrocuprein only; 16.5% with ethylhydrocuprein plus serum; 33% with serum only; and 30% in cases treated expectantly. These statistics are not quoted by us in proof of anything, but simply to illustrate the clinical status of the question. With ethylhydrocuprein as with quinin, both time and dose are important elements, and the sufficient dose of quinin is less dangerous than the sufficient dose of optochin.

¹¹ Jour. Exper. Med., 1915, 22, p. 389.

¹² Berlin. klin. Wchnschr., 1913, 50, p. 1984.

problems not as yet touched on by others, it was deemed best, both for completeness of record and to ensure consistency of methods and comparability of results throughout, to begin independently and on our own lines. In so far as we confirm previous observations, that is worth while. In so far as we differ in results from our predecessors, the question of the cause of this difference is opened.

We have had of course the benefit of Morgenroth's addition to the list of chemical pneumococcides available; and part of our task has been to make various comparisons between this substance and substances derived more directly from the cinchona bark. Besides optochin and its chemical precursor hydroquinin (methylhydrocuprein) and their respective hydrochlorids, the chemicals chosen for study were the alkaloid quinin and its monobasic and dibasic salts with hydrochloric, hydrobromic, and sulfuric acids; its salicylate, lactate, arsenate, valerianate, and tannate; and the double salt of hydrochloric acid with quinin and urea. This has seemed to provide a sufficiently wide variety to permit the development of all important factors of modification. (For most of the quinin salts we are indebted to Messrs. Rosengarten and Co., who generously contributed these for experimental purposes.)

For comparison and cross tests, arsenobenzol, phenol, and mercuric chlorid were used.

Also, urea hydrochlorid, hydrochloric acid, sodium salicylate, and salicylic acid were studied by the same methods, as part of a more extended investigation into the relative influence of acid radical, basic radical, and the molecule as a whole.

Types I, II, and III of the pneumococcus were selected as the microorganisms for investigation; and for comparison and cross-testing, *B. typhosus* and *S. aureus*. (We are indebted to Dr. Rufus Cole for several of the strains of pneumococci used in these studies.)

After it had been found that except for the slightly greater resistance of Type III, there was scarcely as much difference in the results regularly obtained with the different type cultures, as at times with different individual cultures of any one type, certain observations were made with one type only (usually I or II). So, too, some tests (or tabulated reports of results) were confined to a few of the more important chemicals, after it had been found that for the special purpose in view, other differences were relatively unimportant.

In every instance, however, a sufficient number of observations, and in some tests, a multitudinous number, were made. The technic of the

various tests employed is described briefly. For more detailed descriptions and discussion we refer to the second paper of this series.¹³

Morgenroth and Levy¹⁴ found that ethylhydrocuprein possessed considerable protective power against experimental pneumococcal infections in mice and a certain degree of curative action on such infections when established. Wright and his associates¹⁵ found that a dilution of ethylhydrocuprein 1:400,000 in serum killed pneumococci, while a dilution of 1:800,000 inhibited their growth. Practically the same results were observed in a menstruum of water. Wright used a pipet method, and his figures are therefore unusually high, the number of cocci being rather small, that is, only those of a 24-hour broth culture which adhered to a small portion of the wall of a capillary pipet. Lysol, creosote, and guaiacol tested in the same way, killed pneumococci in serum in dilutions of from 1:500 to 1:12,500; while in a menstruum of water these substances were active in dilutions ranging from 1:62,500 to 1:500,000. Tugendreich and Russo¹⁶ found optochin in dilutions in water up to 1:16,000 bactericidal for pneumococci after an exposure of 3 hours at room temperature. Moore¹⁷ found that ethylhydrocuprein hydrochlorid in very high dilution, *in vitro*, inhibited the growth of pneumococci and in lower dilutions, killed them. No constant or considerable differences were seen in these effects on typical representatives of the four groups of pneumococci. Quinin hydrochlorid was found more bactericidal for other microorganisms than for pneumococci, but proved less powerful in this respect than optochin. (Our results lead to a different conclusion as regards *B. typhosus* and *S. aureus*.) More recent observations regarding the bactericidal action of quinin in general are reported by Taylor,¹⁸ who found quinin hydrochlorid 10 times more effective than phenol against *B. aerogenes-capsulatus*, and who accordingly recommends the use of this compound in the treatment of infected wounds.

In the studies here reported, we had in view (1) to determine by various methods the degree of activity and relative value of different cinchona derivatives as pneumococcidal agents *in vitro*; (2) to study the specificity of the action of quinin compounds and the other cinchona derivatives on pneumococci *in vitro* by cross-bactericidal tests with other disinfectants and other microorganisms; (3) to study the influence of acid radicals and other components of the molecules as well as of the quinin base; and (4) to study the influence of solubility.

RESULTS WITH THE CENTRIFUGATION METHOD

With this method large numbers of pneumococci were exposed to the action of the bactericide for an hour at 37 C., after which the cocci were removed, washed once by centrifugation, and then cultured. Only

¹³ Kolmer, Cohen, and Heist, *Jour. Infect. Dis.*, 1917, 20, p. 61.

¹⁴ *Berl. klin. Wchnschr.*, 1911, 48, pp. 1561, 1650, 1779, 1983.

¹⁵ *Lancet*, 1912, 11, pp. 1633, 1701.

¹⁶ *Ztschr. f. Immunitätsf.*, 1913, 19, p. 165.

¹⁷ *Jour. Exper. Med.*, 1915, 22, p. 269.

¹⁸ *Lancet*, 1905, 11, p. 538.

those compounds could be used which gave a clear solution, the less soluble salts being unsuitable for observation by this method because of the impossibility of separating the cocci from the sediment of undissolved drug. Since this test makes use (1) of large numbers of microorganisms, (2) of a high concentration of the drug, and (3) of a short time exposure, it may be regarded as germicidal in the strict sense.

The results of a number of these tests are summarized in Table 1.

TABLE 1
RESULTS OF BACTERICIDAL TESTS BY THE CENTRIFUGATION METHOD

Substance	Dilution Killing in 1 Hour		
	Type I	Type II	Type III
Ethylhydrocuprein hydrochlorid	1:800	1:700	1:700
Quinin and urea hydrochlorid.....	1:100	1:200	1:150
Hydroquinin hydrochlorid	1:100	1:100	1:80
Quinin bisulfate	1:100	1:80	1:80
Quinin dihydrochlorid	1:100	1:120	1:100
Quinin hydrobromid	1:100	1:100	1:100

The various salts of quinin proved bactericidal for all types in dilution of about 1:100 (least active 1:80; most active 1:200). Methylhydrocuprein (hydroquinin) hydrochlorid had about the same germicidal value as the quinin compounds.

The compound of quinin and urea hydrochlorid was slightly more active than the other quinin compounds against Type II (1:200) and Type III (1:150).

Ethylhydrocuprein hydrochlorid, however, proved 3.5 to 8 times as active as the most active of the quinin salts (bactericidal in dilution of 1:700 and 1:800).

The results with cultures of Types I, II, and III were in general approximately the same. Type III (*Pneumococcus mucosus*), however, in several instances proved somewhat more difficult to kill.

RESULTS WITH A PIPET METHOD

According to Wright and his associates the bactericidal power of ethylhydrocuprein hydrochlorid remains almost the same in a menstruum of serum as in salt solution. From the viewpoint of bactericidal chemotherapy this fact is important, since as Wright points out, a substance which is actively germicidal in serum may be presumed to have but slight tendency for an inactivating union with the protein of the body fluids.

With the pipet method, which permits this comparison to be made readily, we tested at 37 C., a number of compounds in a menstruum of sterile serum and in a menstruum of salt solution, using a culture of pneumococcus belonging to Type I. Altho we did not confirm Wright's observation as to retention by optochin of an activity in serum approximately equal to that in salt solution, we found its bactericidal power in serum to be high.

The results of these tests are summarized in Table 2.

TABLE 2
RESULTS OF COMPARATIVE BACTERICIDAL TESTS IN SERUM AND SALT SOLUTION
BY THE PIPET METHOD

Substance	Dilution Killing in 30 Minutes	
	In Serum	In Salt Solution
Ethylhydrocuprein.....	1:20,000	1:100,000
Quinin and urea hydrochlorid.....	1:200	1:250
Hydroquinin hydrochlorid.....	1:500	1:750
Quinin bisulfate.....	1:300	1:500
Quinin hydrochlorid.....	1:100	1:500
Quinin tannate.....	1:30	1:50
Quinin dihydrobromid.....	1:1000	1:500
Quinin salicylate.....	1:300	1:500
Phenol.....	1:150	1:300
Mercuric chlorid.....	1:1000	1:10,000

While ethylhydrocuprein hydrochlorid is still highly active in a menstruum of serum, its bactericidal power is reduced to one-fifth part of that shown in normal salt solution.

The pneumococcidal power of most of the quinin salts is likewise lessened in serum, as compared with that in salt solution, but to nothing like the same degree as that of ethylhydrocuprein; so that the discrepancy in their general bactericidal power—while it appears to be much greater by this method than by any other of those used in this series of studies—is much less apparent in serum than in salt solution. For example, the ratio of germicide activity between quinin and urea hydrochlorid and ethylhydrocuprein is 250:100,000 in salt solution (i. e., 1:400), while in serum it is 200:20,000 (i. e., 1:100). In other words, the reduction in the power of optochin is 4 times that of the carbamide compound.

Surprisingly, one salt (the dihydrobromid) proved twice as bactericidal in serum as in salt solution, showing one-half the activity of optochin in the former, as against one-two-hundredth in the latter. This exceptional result suggests further study in several directions.

The pneumococcidal activity of phenol was reduced in serum to one-half, and that of mercuric chlorid to one-tenth of the power shown in salt solution.

The high bactericidal power of ethylhydrocuprein for pneumococci is evident in these tests, but its superiority and that of the quinin compounds in this connection over phenol and mercuric chlorid are far more striking in the antiseptic test, in which the drugs in low concentration are permitted to act on the pneumococci over a longer period.

RESULTS WITH THE COMBINED IN-VITRO-VIVO METHOD

In this method large numbers of highly virulent pneumococci belonging to Type I were exposed to high concentrations of ethylhydrocuprein and other cinchona derivatives at 37 C. in a menstruum of normal salt solution, and the degree of bactericidal activity of these cinchona cultures was tested in white mice by intraperitoneal injection of small amounts at different intervals up to 1 hour. It yielded fairly constant and decisive results, which are summarized in Tables 3 and 4.

TABLE 3
RESULTS OF BACTERICIDAL TESTS BY THE COMBINED IN-VITRO-VIVO METHOD *

Substance	Dilution	Exposure in Minutes				
		5	15	30	45	60
Ethylhydrocuprein hydrochlorid..	1:1000	—	—	—	—	—
	1:2000	+	—	—	—	—
	1:10000	+	+	+	+	+
Quinin and urea hydrochlorid....	1:100	—	—	—	—	—
	1:500	+	+	—	—	—
	1:1000	+	+	+	+	+
Hydroquinin hydrochlorid.....	1:100	—	—	—	—	—
	1:500	+	+	+	—	—
	1:1000	+	+	+	+	—
Quinin bisulfate.....	1:200	+	—	—	—	—
	1:1000	+	+	+	+	+
Quinin hydrochlorid.....	1:100	—	—	—	—	—
	1:500	—	—	—	—	—
	1:1000	+	—	—	—	—
Quinin lactate.....	1:200	—	—	—	—	—
	1:500	+	+	—	—	—
	1:1000	+	+	+	+	+
Quinin salicylate.....	1:100	—	—	—	—	—
	1:500	+	—	—	—	—
	1:1000	+	+	+	+	—
Quinin arsenate.....	1:200	—	—	—	—	—
	1:500	+	+	—	—	—
	1:1000	+	+	+	+	+
Quinin (alkaloid).....	1:100	—	—	—	—	—
	1:200	—	—	—	—	—
	1:500	+	+	—	—	—
	1:1000	+	+	+	+	+
Controls.....		+	+	+	+	+

* *Pneumococcus* Type I used, of which the M. L. D. for mice was 0.000001 e.c. of a 24-hour broth culture.

+ = animal died. — = animal living (48 hours).

TABLE 4
RESULTS OF BACTERICIDAL TESTS BY THE COMBINED IN-VITRO-VIVO METHOD
(PNEUMOCOCCUS TYPE I).

Substance	Dilution	Exposure in Minutes				
		5	15	30	45	60
Phenol.....	1:100	—	—	—	—	—
	1:200	+	+	+	—	—
	1:500	+	+	+	+	+
Bichlorid of mercury.....	1:100	—	—	—	—	—
	1:200	+	+	—	—	—
	1:500	+	+	+	+	—
Controls.....		+	+	+	+	+

+ = animal died. — = animal living (48 hours).

In certain instances the quinin salts were used in concentrations greater than their maxima of solubility in salt solution, but the presence of undissolved particles did not appear to interfere with the regularity of the results.

Altho the mice used in these experiments were kept under observation for 6 days, the tables were prepared according to the results observed within 48 hours. After this time the results were irregular; some of the animals, as shown by necropsy (see third paper of this series), succumbing to the toxic effects of the drugs rather than to an infection with pneumococci.

Ethylhydrocuprein hydrochlorid proved 10 times more bactericidal in the 5-minute period of exposure, and from 5 to 10 times more active in the 15-minute period, than the majority of the quinin compounds, phenol, and mercuric chlorid. Exposure of the culture (Type I) to a solution of 1:2000 for 15 minutes, or of 1:1000 for 5 minutes, was sufficient to prevent infection of the animals.

Quinin hydrochlorid, however, in both the 5-minute and 15-minute periods, was half as active as optochin; the alkaloid quinin and quinin arsenate one-fifth as active.

After an exposure of 30 minutes, and still more after one of 60 minutes, the difference between the bactericidal activity of ethylhydrocuprein and that of the quinin compounds in general was markedly lessened; the former being only 2 to 4 times more active than the latter. The compound of quinin and urea hydrochlorid was germicidal in dilution of 1:500 after 30 minutes' exposure, and quinin salicylate in 1:500 after 15 minutes' exposure.

TABLE 5
RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD (PNEUMOCOCCUS TYPE I)

Salts	Percent- age of Alkaloid	Dilutions							
		1:1000	1:2000	1:4000	1:5000	1:8000	1:10,000	1:16,000	1:20,000
Quinin tannate.....	20.0	—	—	—	—	—	—	—	—
Quinin and urea hydrochlorid....	58.0	—	—	—	—	—	—	+	+
Quinin bisulfate.....	59.1	—	—	—	—	—	—	—	—
Quinin dihydrobromid.....	60.0	—	—	—	—	—	—	—	—
Quinin arsenate.....	69.4	—	—	—	—	—	—	—	—
Quinin salicylate.....	70.1	—	—	—	—	—	—	—	—
Quinin dihydrochlorid.....	72.0	—	—	—	—	—	—	—	—
Quinin valerinate.....	73.0	—	—	—	—	—	—	—	—
Quinin sulfate.....	73.5	—	—	—	—	—	—	—	—
Quinin hydrobromid.....	76.0	—	—	—	—	—	—	—	—
Quinin phosphate.....	76.2	—	—	—	—	—	—	—	—
Quinin lactate.....	78.2	—	—	—	—	—	—	—	—
Quinin hydrochlorid.....	81.8	—	—	—	—	—	—	—	—
Quinin alkaloid.....	100.0	—	—	—	—	—	—	—	—
Ethylhydrocuprein hydrochlorid.....	75-80	—	—	—	—	—	—	—	—
Hydroquinin hydrochlorid.....	90.8	—	—	—	—	—	—	—	—

+ = growth. — = sterility.

TABLE 6
RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD (PNEUMOCOCCUS TYPE II)

Salts	Percent- age of Alkaloid	Dilutions							
		1:1000	1:2000	1:4000	1:5000	1:8000	1:10,000	1:16,000	1:20,000
Quinin tannate.....	20.0	—	—	—	—	—	—	—	—
Quinin and urea hydrochlorid....	58.0	—	—	—	—	—	—	—	—
Quinin bisulfate.....	59.1	—	—	—	—	—	—	—	—
Quinin dihydrobromid.....	60.0	—	—	—	—	—	—	—	—
Quinin arsenate.....	69.4	—	—	—	—	—	—	—	—
Quinin salicylate.....	70.1	—	—	—	—	—	—	—	—
Quinin dihydrochlorid.....	72.0	—	—	—	—	—	—	—	—
Quinin valerinate.....	73.0	—	—	—	—	—	—	—	—
Quinin sulfate.....	73.5	—	—	—	—	—	—	—	—
Quinin hydrobromid.....	76.0	—	—	—	—	—	—	—	—
Quinin phosphate.....	76.2	—	—	—	—	—	—	—	—
Quinin lactate.....	78.2	—	—	—	—	—	—	—	—
Quinin hydrochlorid.....	81.8	—	—	—	—	—	—	—	—
Quinin alkaloid.....	100.0	—	—	—	—	—	—	—	—
Ethylhydrocuprein hydrochlorid.....	75-80	—	—	—	—	—	—	—	—
Hydroquinin hydrochlorid.....	90.8	—	—	—	—	—	—	—	—

+ = growth. — = sterility.

TABLE 7
RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD (PNEUMOCOCCUS TYPE III)

Salts	Percent- age of Alkaloid	Dilutions							
		1:666	1:1000	1:2000	1:4000	1:5000	1:8000	1:10,000	1:16,000
Quinin tannate.....	20.0	—	—	—	—	—	+	+	+
Quinin and urea hydrochlorid....	58.0	—	—	—	—	—	—	—	—
Quinin bisulfate.....	59.1	—	—	—	—	—	—	—	—
Quinin dihydrobromid.....	60.0	—	—	—	—	—	—	—	—
Quinin arsenate.....	69.4	—	—	—	—	—	—	—	—
Quinin salicylate.....	70.1	—	—	—	—	—	—	—	—
Quinin dihydrochlorid.....	72.0	—	—	—	—	—	—	—	—
Quinin valerinate.....	73.0	—	—	—	—	—	—	—	—
Quinin sulfate.....	73.5	—	—	—	—	—	—	—	—
Quinin hydrobromid.....	76.0	—	—	—	—	—	—	—	—
Quinin phosphate.....	76.2	—	—	—	—	—	—	—	—
Quinin lactate.....	78.2	—	—	—	—	—	—	—	—
Quinin hydrochlorid.....	81.8	—	—	—	—	—	—	—	—
Quinin alkaloid.....	100.0	—	—	—	—	—	—	—	—
Ethylhydrocuprein hydrochlorid.....	75-80	—	—	—	—	—	—	—	—
Hydroquinin hydrochlorid.....	90.8	—	—	—	—	—	—	—	—

+ = growth. — = sterility.

Dilutions									
1:50,000	1:80,000	1:100,000	1:200,000	1:800,000	1:1,000,000	1:1,500,000	1:2,000,000	1:3,000,000	1:4,000,000
+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+
—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—
—	+	—	+	+	+	+	+	+	+
—	+	+	+	+	+	+	+	+	+
—	+	+	+	+	+	+	+	+	+
—	+	+	+	+	+	+	+	+	+
—	—	—	—	+	+	+	+	+	+
—	—	—	—	+	+	+	+	+	+
—	—	—	—	+	+	+	+	+	+
—	—	—	—	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+
—	—	+	—	—	—	+	+	+	+
—	—	+	+	+	+	+	+	+	+

Dilutions									
1:50,000	1:80,000	1:100,000	1:200,000	1:800,000	1:1,000,000	1:1,500,000	1:2,000,000	1:3,000,000	1:4,000,000
+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+
-	-	-	+	+	+	+	+	+	+
-	-	-	-	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+
-	-	+	+	+	+	+	+	+	+
-	-	-	+	+	+	+	+	+	+
-	-	-	+	+	+	+	+	+	+
-	-	+	+	+	+	+	+	+	+
-	+	+	-	+	+	+	+	+	+
-	+	+	+	+	-	+	-	+	+

[illegible]

RESULTS WITH THE ANTISEPTIC METHOD

In the method which we have termed 'antiseptic' small numbers of the microorganisms are exposed to high dilutions of the disinfectant over a long period of time. This form of test has brought out more clearly the efficacy and high degree of specificity of cinchona derivatives as pneumococides.

A large number of such experiments have been made with a number of quinin compounds, with optochin, and with hydroquinin, on cultures of pneumococci belonging to Types I, II, and III, the results being summarized in Tables 5, 6, and 7.

All these tests were conducted with solutions of the drugs in broth and at a temperature of 37 C. The results tabulated were noted after an 8-day period of observation. Had the readings been recorded as observed at the end of the first 24 hours, the values shown would have been much higher, since many tubes of the lower dilutions were apparently sterile at this time, which later evidenced the growth of pneumococci. No tabulation of this retarding effect is here presented, but a record of the fact is thus made.

Ethylhydrocuprein hydrochlorid inhibits the multiplication of pneumococci belonging to the first three serologic types, in dilutions varying from 1:1,000,000 to 1:2,000,000 (Tables 5, 6, and 7).

The results with the quinin salts differ considerably from one another, the inhibiting dilutions ranging from 1:5000 and 1:20,000 (quinin tannate) to 1:100,000 and 1:200,000 (quinin hydrobromid), or from one-two-hundredth to one-tenth the power of ethylhydrocuprein. Quinin alkaloid ranges from 1:20,000 against Type III to 1:50,000 against Type II.

According to the calculated amounts of quinin contained in the various salts tested (see Tables 5, 6, and 7) there is no definite relation apparent between the percentage of alkaloid present and the antiseptic power of the compound. Tests to determine the antiseptic value of the various acid radicals and other suggested factors of modification are under way, but are not sufficiently numerous, as yet, to warrant conclusions.

The quinin and urea hydrochlorid compound was relatively low in antiseptic value in these tests (1:10,000 for Type I, 1:20,000 for Type II, 1:16,000 for Type III), altho with other methods, or germicidal tests as we have called them, in which low dilutions were used with short exposure — as also with the plating method, later described.

which gives both antiseptic and germicidal values — this double salt yielded results which compared favorably with, and in some instances were much better than, those with other quinin compounds. (This tends to confirm the view that the bactericidal effect is neither the only nor the chief value of quinin compounds in general, and of the carbamide in particular, as therapeutic agents in pneumonia.) Urea hydrochlorid

TABLE 8

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD (EFFECT OF UREA HYDROCHLORID ON PNEUMOCOCCI'S TYPE I)

No.	Culture	Broth	Dilutions	Results (Days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:100	+	+	+	+	+	+	+	+
2			1:150	+	+	+	+	+	+	+	+
3			1:200	+	+	+	+	+	+	+	+
4			1:300	+	+	+	+	+	+	+	+
5			1:500	+	+	+	+	+	+	+	+
6			1:1000	+	+	+	+	+	+	+	+
7			Control	+	+	+	+	+	+	+	+

+ = growth.

TABLE 9

SUMMARY OF RESULTS OF BACTERICIDAL TESTS ON DIFFERENT TYPES OF PNEUMOCOCCI BY THE ANTISEPTIC METHOD

Substance	Dilutions in Which the Various Pneumococci Were Killed		
	Type I	Type II	Type III
Ethylhydrocuprein hydrochlorid.....	1:1,000,000	1:2,000,000	1:1,500,000
Hydroquinin hydrochlorid.....	1: 80,000	1: 50,000	1: 20,000
Quinin and urea hydrochlorid.....	1: 10,000	1: 20,000	1: 16,000
Quinin (alkaloid).....	1: 20,000	1: 50,000	1: 20,000
Quinin bisulfate.....	1: 80,000	1: 80,000	1: 100,000
Quinin tannate.....	1: 20,000	1: 20,000	1: 5,000
Quinin dihydrobromid.....	1: 100,000	1: 100,000	1: 20,000
Quinin arsenate.....	1: 100,000	1: 200,000	1: 100,000
Quinin salicylate.....	1: 50,000	1: 100,000	1: 100,000
Quinin dihydrochlorid.....	1: 50,000	1: 20,000	1: 20,000
Quinin valerate.....	1: 50,000	1: 80,000	1: 16,000
Quinin sulfate.....	1: 200,000	1: 100,000	1: 20,000
Quinin hydrobromid.....	1: 200,000	1: 100,000	1: 20,000
Quinin phosphate.....	1: 100,000	1: 80,000	1: 20,000
Quinin lactate.....	1: 100,000	1: 100,000	1: 20,000
Quinin hydrochlorid.....	1: 100,000	1: 200,000	1: 100,000

was found without demonstrable bactericidal properties over pneumococci in dilutions as low as 1: 100 (Table 8). tho Synmers and Kirk¹⁰ found that urea was active as a germicide in very low dilutions and advocated it in the treatment of infected wounds. In so far as the double hydrochlorid of quinin and urea is concerned, the bactericidal activity on pneumococci is to be ascribed to the quinin salt.

¹⁰ Lancet, 1915, 11, p. 1237.

The inhibiting influence of the cinchona derivatives was about the same on Types I and II of pneumococci. With Type III somewhat lower values were found; that is, this type was apparently less susceptible to the action of quinin (Table 9). Because, however, of the irregularities exhibited by different cultures belonging to the same type we are not inclined to lay too much stress on these differences (see Table 14).

RESULTS WITH A PLATING METHOD

A summary of the results observed with a plating method employing ethylhydrocuprein hydrochlorid and 4 other cinchona derivatives and a culture of pneumococcus belonging to Type I, is shown in Table 10.

The bactericide in varying dilutions was allowed to remain in contact with the cocci for a period of 24 hours at 37 C. Then subcultures were made in 10 c.c. of blood agar.

TABLE 10

SUMMARY OF RESULTS OF BACTERICIDAL TESTS BY THE PLATING METHOD (THE BACTERICIDAL ACTION OF CINCHONA DERIVATIVES ON PNEUMOCOCCUS TYPE I)

Substance	Highest Dilution Causing	
	Inhibition	Sterility
Ethylhydrocuprein hydrochlorid.....	1:1,000,000	1:40,000
Hydroquinin hydrochlorid.....	1: 60,000	1: 7000
Quinin and urea hydrochlorid.....	1: 60,000	1: 6000
Quinin bisulfate.....	1: 40,000	1: 5000
Quinin hydrochlorid.....	1: 40,000	1: 4000
Urea hydrochlorid.....	Not in 1:100	Not in 1:100

The particular advantage of this method lies in its showing under identical conditions both the inhibiting, or antiseptic, and the killing, or bactericidal, dose of the chemical studied.

The inhibiting dose of the various compounds (exposure 24 hours) was from 8 to 25 times less than the bactericidal dose, ethylhydrocuprein proving by far the most active (1:1,000,000 and 1:40,000), and hydroquinin and quinin and urea hydrochlorid next in both inhibiting (1:60,000) and sterilizing power (1:7000 and 1:6000) in all these tests (Table 10).

INFLUENCE OF PERIOD OF EXPOSURE

The evidence at hand indicates that quinin and its derivatives (under which term we may broadly include optochin as well as hydroquinin) act somewhat slowly on microorganisms and demonstrate

more clearly their specificity for the pneumococcus in the antiseptic tests (with long exposures) than in the germicidal tests (in which the exposure is brief). This fact is further illustrated by the results presented in Tables 2, 4, and 13. In all of the tests there set forth, phenol and mercuric chlorid were used as standards of comparison; and were shown to rank fairly high as germicides for the pneumococcus (Tables 2 and 4), but to fall far below the quinin derivatives in the antiseptic tests (Table 13).

THE SPECIFICITY OF THE BACTERICIDAL ACTION OF ETHYLHYDROCUPREIN
HYDROCHLORID AND OTHER CINCHONA (QUININ)
DERIVATIVES ON PNEUMOCOCCI IN VITRO

The specificity of the bactericidal action of cinchona derivatives on pneumococci was studied by cross-testing their efficacy against *B. typhosus* and *S. aureus*; while also testing the bactericidal activity of phenol, mercuric chlorid, arsenobenzol, and other chemicals on pneumococci.

In a general way the results of these tests show conclusively that, in long-exposure tests at least, the quinin derivatives possess a high degree of specific pneumococcidal activity.

The results of tests with various quinin compounds and optochin, using the technic of the antiseptic method, and *B. typhosus* and *S. aureus* as the test microorganisms, are shown in Tables 11 and 12.

In a general way the results observed with both microorganisms and all the quinin salts were similar; that is, the inhibiting dilution usually ranged from 1:1000 to 1:2000. Ethylhydrocuprein hydrochlorid was slightly the more bactericidal for *B. typhosus* (1:2500), and decidedly the more so for *S. aureus* (1:5000) (Tables 11 and 12).

The value of ethylhydrocuprein as a bactericide was from 200 to 400 times greater for the pneumococcus than for *B. typhosus* and the staphylococcus; the various quinin derivatives showed a bactericidal activity against the pneumococcus ranging from 5 to 50 times greater than against the other microorganisms (Table 13).

Arsenobenzol in dilution of 1:2500 was effective against pneumococci, and in dilutions of from 1:1000 to 1:2000, against *B. typhosus* and staphylococci, but far inferior to the quinin derivatives as a pneumococcide. Likewise phenol, mercuric chlorid, and sodium salicylate proved far less active than the cinchona derivatives as pneumococicides.

TABLE 11

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD (THE BACTERICIDAL ACTION OF QUININ SALTS AND DERIVATIVES ON *B. TYPHOSUS*)

Substance	Percent- age of Alkaloid	Dilutions						
		1:100	1:200	1:300	1:400	1:500	1:600	1:700
Quinin tannate.....	20.0	—	—	—	—	—	—	—
Quinin and urea hydrochlorid...	58.0	—	—	—	—	—	—	—
Quinin bisulfate.....	59.1	—	—	—	—	—	—	—
Quinin dihydrobromid.....	60.0	—	—	—	—	—	—	—
Quinin arsenate.....	69.4	—	—	—	—	—	—	—
Quinin salicylate.....	70.1	—	—	—	—	—	—	—
Quinin dihydrochlorid.....	72.0	—	—	—	—	—	—	—
Quinin valerate.....	73.0	—	—	—	—	—	—	—
Quinin sulfate.....	73.5	—	—	—	—	—	—	—
Quinin hydrobromid.....	76.6	—	—	—	—	—	—	—
Quinin phosphate.....	76.2	—	—	—	—	—	—	—
Quinin lactate.....	78.2	—	—	—	—	—	—	—
Quinin hydrochlorid.....	81.8	—	—	—	—	—	—	—
Quinin alkaloid.....	100.0	—	—	—	—	—	—	—
Ethylhydrocuprein hydrochlorid	90.8	—	—	—	—	—	—	—
Hydroquinin hydrochlorid.....	75-80	—	—	—	—	—	—	—
Arsenobenzol.....	—	—	—	—	—	—	—

+ = growth. — = sterility.

TABLE 12

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD (THE BACTERICIDAL ACTION OF QUININ SALTS AND DERIVATIVES ON *STAPHYLOCOCCUS AUREUS*)

Substance	Percent- age of Alkaloid	Dilutions						
		1:100	1:200	1:300	1:400	1:500	1:600	1:700
Quinin tannate.....	20.0	—	—	—	—	—	—	—
Quinin and urea hydrochlorid...	58.0	—	—	—	—	—	—	—
Quinin bisulfate.....	59.1	—	—	—	—	—	—	—
Quinin dihydrobromid.....	60.0	—	—	—	—	—	—	—
Quinin arsenate.....	69.4	—	—	—	—	—	—	—
Quinin salicylate.....	70.1	—	—	—	—	—	—	—
Quinin dihydrochlorid.....	72.0	—	—	—	—	—	—	—
Quinin valerate.....	73.0	—	—	—	—	—	—	—
Quinin sulfate.....	73.5	—	—	—	—	—	—	—
Quinin hydrobromid.....	76.6	—	—	—	—	—	—	—
Quinin phosphate.....	76.2	—	—	—	—	—	—	—
Quinin lactate.....	78.2	—	—	—	—	—	—	—
Quinin hydrochlorid.....	81.8	—	—	—	—	—	—	—
Quinin alkaloid.....	100.0	—	—	—	—	—	—	—
Ethylhydrocuprein hydrochlorid	75-80	—	—	—	—	—	—	—
Hydroquinin hydrochlorid.....	—	—	—	—	—	—	—
Arsenobenzol.....	—	—	—	—	—	—	—

+ = growth. — = sterility.

Hydrochloric and salicylic acids proved so much less bactericidal than their respective salts with quinin that so far as these tests go, the base is evidently the active constituent.

TABLE 13
SUMMARY OF RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD (EFFECT OF QUININ DERIVATIVES AND OTHER DISINFECTANTS ON VARIOUS MICROORGANISMS)

Substance	Dilutions that Killed		
	Pneumococcus Type I	B. Typhosus	S. Aureus
Ethylhydrocuprein hydrochlorid.....	1:1,000,000	1:2500	1:5000
Quinin and urea hydrochlorid.....	1: 10,000	1:2000	1:2000
Hydroquinin hydrochlorid.....	1: 50,000	1:2000	1:2000
Quinin bisulfate.....	1: 80,000	1:1000	1:2000
Quinin dihydrobromid.....	1: 100,000	1:2000	1:1000
Quinin salicylate.....	1: 50,000	1:1000	1:1000
Quinin tannate.....	1: 20,000	1:1000	1:1000
Quinin (alkaloid).....	1: 20,000	1:2000	1:1000
Arsenobenzol.....	1: 2500	1:2000	1:1000
Phenol.....	1: 1000	1: 125	1: 80
Bichlorid of mercury.....	1: 5000	1:20,000	1:20,000
Urea hydrochlorid.....	Not in 1:100	Not in 1:100	Not in 1:100
Salicylic acid.....	1: 1000	0*	0
Sodium salicylate.....	1: 300	0	0
Hydrochloric acid.....	1: 100	0	0

* 0 = not tested.

TABLE 14
VARIATION IN RESISTANCE TO BACTERICIDAL AGENTS AMONG CULTURES OF THE SAME TYPE OF PNEUMOCOCCUS

Substance	Method	Highest Dilution that Killed			
		Type I		Type II	
		A	B	A	B
Ethylhydrocuprein hydrochlorid.....	Antiseptic.....	1:1,000,000	1:1,500,000	1:2,000,000	1:3,200,000
	Plating.....	1: 40,000	1: 20,000	1: 80,000	1: 120,000
	Pipet*.....	1: 100,000	1: 200,000	1: 150,000	1: 120,000
Hydroquinin hydrochlorid.....	Antiseptic.....	1: 80,000	1: 60,000	1: 50,000	1: 100,000
	Plating.....	1: 6000	1: 14,000	1: 8000	1: 10,000
	Pipet.....	1: 500	1: 400	1: 600	1: 1000
Quinin and urea hydrochlorid.....	Antiseptic.....	1: 10,000	1: 40,000	1: 20,000	1: 30,000
	Plating.....	1: 6000	1: 8000	1: 8000	1: 8000
	Pipet.....	1: 200	1: 200	1: 250	1: 400

* In serum in all tests.

VARIATION IN THE BACTERICIDAL ACTION OF CINCHONA DERIVATIVES ON DIFFERENT STRAINS OF PNEUMOCOCCI BELONGING TO THE SAME TYPE

Moore found that there were practically no differences among pneumococci belonging to the four serologic types in their resistance

to the bactericidal action of ethylhydrocuprein and quinin hydrochlorid. As mentioned, we have observed on occasions that Type III (*Pneumococcus mucosus*) was slightly more difficult to kill than Types I and II.

A number of experiments, after various methods, with different cultures belonging to the same serologic types, developed, however, considerable variations in resistance. A few of these experiments with Types I and II and 3 cinchona derivatives, are summarized in Table 14.

As shown in the table, the variations in resistance were frequently of considerable magnitude. In all our preceding tables, therefore, we have taken the lowest figures. We are unable to offer an adequate explanation of these differences, but experiments have suggested that freshly isolated strains, and strains freshly passed through mice with marked increase of virulence, are more resistant than the same strains carried for some time in artificial culture media.

CONCLUSIONS

Certain cinchona derivatives — quinin, hydroquinin (methylhydrocuprein), optochin (ethylhydrocuprein), and their salts — tested by various methods, exert a very high bactericidal power on pneumococci in vitro, ethylhydrocuprein being by far the most active. Similar studies of other cinchona alkaloids with their compounds and modifications are to be desired.

The results of bactericidal tests numerically expressed, vary greatly according to the technic employed and according to such factors as temperature, duration of exposure, and vigor and virulence of the microorganisms. Stated and standardized methods are therefore necessary, since only those results that are obtained by the same technic, are susceptible of direct comparison.

While the various cinchona derivatives studied exhibit considerable differences in relative bactericidal value, it is probable that the power of any one of them is about the same for all types of pneumococci, tho in several instances Type III (*Pneumococcus mucosus*) was found somewhat more resistant than the other two types studied. The fact that the different quinin compounds do not, as might have been presumed, vary in bactericidal activity according to their respective alkaloidal strengths, makes needful a further investigation of the relative influence of acid and basic radicals and of the molecule as a whole.

Different cultures of pneumococci belonging to the same serologic type exhibit varying degrees of resistance to bactericidal agents. It is probable that this variation bears a relation to virulence, and more

particularly to whether the strain has been freshly isolated from lesions or has been cultivated for a prolonged period on an artificial medium.

The cinchonics studied act slowly on pneumococci, the best bactericidal and inhibiting values (that is, the most marked activity of high dilutions — from about 1:10,000 to 1:2,000,000) being shown when the number of microorganisms is small and the exposure relatively prolonged. Nevertheless, even with brief exposures (5 to 60 minutes) and large numbers of virulent microorganisms, moderately high concentrations (from 1:100 to 1:2000) are germicidal.

Cross bactericidal tests — employing, on the one hand, quinin and optochin compounds, with cultures of *B. typhosus* and of a staphylococcus, and on the other hand, arsenobenzol, phenol, mercuric chlorid, sodium salicylate, and other substances, with cultures of pneumococci — show that the cinchona derivatives possess a high degree of specific pneumococcidal activity.

The pneumococcidal activity of ethylhydrocuprein hydrochlorid was reduced to one-fifth or sometimes one-tenth part in a menstruum of serum, as compared with that shown in normal salt solution. The activity of the majority of the quinin salts tested was, relatively, but slightly reduced in serum. The superiority of optochin over quinin was thus, to a certain extent, lessened. The activity of phenol in serum was reduced to one-half, and that of mercuric chlorid to one-tenth, of their respective values in salt solution. For bactericidal chemotherapy it is desirable that agents to be used clinically maintain a high degree of germicidal power in serum, in tests in vitro.

VARIOUS METHODS OF DETERMINING THE BACTERI-
CIDAL ACTION OF SUBSTANCES IN VITRO AND
THEIR RELATION TO THE CHEMO-
THERAPY OF BACTERIAL
INFECTIONS *

STUDIES IN PNEUMONIA, II

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In the rational development of the chemotherapy of bacterial and protozoan infections it is desirable, if possible, to commence experimental work with a substance or substances possessing some definite destructive effect on the microparasite under study. This parasitotropic effect may be apparent only in test-tube experiments; in such event, an effort is made to lower the toxicity or organotropic effect of the substance, with, or even without, an increase of its parasitotropic power, in order that it may be administered in such quantity as will exert in the living animal an inhibitory or killing action on the microparasite under study without injury to the host.

The ultimate aim in bactericidal chemotherapy is the discovery or the rational and systematic development by synthesis, of a substance that is strictly monotropic; that is, one possessing a selective affinity for the protoplasm of a particular microparasite and exerting a specific killing effect on that organism. Thus far this desideratum has not been achieved; as even arsenobenzol (salvarsan) is not strictly parasitotropic for *Spirochaeta pallidum*, but possesses also a marked parasitropism for other spirochetes and other protozoa, notably various trypanosomes. While the development or discovery of substances which are markedly bacteriotropic or protozootropic in general constitutes an important advance, the ultimate aim, as stated, should be the synthesis of a strictly monotropic substance. It is suggested that further studies with arsenobenzol tending to increase its parasitotropic effect on spirochetes alone and *Spirochaeta pallidum* in particular, will still further increase the therapeutic efficacy of this drug.

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'LEADS' IN CHEMOTHERAPEUTIC RESEARCH

In the present state of our knowledge of chemotherapy, chance or accidental discovery must play an important rôle in the discovery of a 'lead.' Substances are to be selected or prepared on as systematic a basis as possible, and tried out by actual experiment; those yielding encouraging results are then subjected to various systematic modifications with experimental trial of the new compounds. In this manner chemotherapeutic research proves to be costly and laborious, as amply demonstrated by the prolonged and costly series of experiments directed by Ehrlich, which resulted in the discovery of arsenobenzol.

In the discovery of leads and the study of new compounds, animal experiments are of primary importance, not only because these are the sole means of determining the organotropic or toxic effects of the compounds, but because they are the sole means of determining the actual parasitotropic or therapeutic effects. In conducting these experiments it is necessary to select a protozoon or bacterium that yields a uniform infection of the animals of not too severe a character, and to reproduce as far as possible the same lesions in the animals as are found in man. The test microparasite should either produce definite lesions easy of detection and study or cause the death of the animal in a given period of time. For studies in bacterial chemotherapy virulent cultures of the pneumococcus are admirably adapted to work with mice and rabbits; in studies of protozoa, *T. equiperdum* or *T. brucei* is valuable, the white rat being used as host.

These experiments, however, are likely to prove costly, and, as in the case of tuberculosis, it may be many weeks or months before results can be determined. Furthermore, special animals such as monkeys or the higher apes may be necessary for the determination of the parasitotropic effect of a substance, as in the case of anterior poliomyelitis and syphilis, in which the particular microparasites fail entirely to infect such animals as rabbits, guinea-pigs, and rats, or, at least, fail to do so with sufficient uniformity. In chemotherapeutic studies in syphilis the rabbit may be employed, altho the infection usually pursues in this animal a brief course tending to spontaneous recovery. For these reasons an effort should be made to train or adapt a race of the particular microparasite under study to survive and multiply in the tissues of an animal easily obtained and handled, so that the abundance of experimental material needed in chemotherapeutic studies on a large scale may be available. In addition to this, as stated,

an effort should be made as far as possible, to reproduce in the experimental animals lesions similar to those found in man. For example, the effect of a drug in the blood of a rabbit or white mouse in a pneumococcus bacteremia must be different from that in the exudate of a consolidated lung in pneumonia of man.

In chemotherapeutic studies on syphilis, experience has shown that a trypanosome may be used, as *T. equiperdum*, or a spirochete other than *S. pallidum*, as *S. gallinarium*, to determine the parasitotropic effect of new compounds as they are produced; because those compounds showing marked effects on these microparasites, as for example arsenobenzol, also produce a profound effect on *S. pallidum* in human and experimental syphilis. It remains to be determined, however, whether similar conditions hold true in the chemotherapy of bacterial infections; that is, whether a compound showing a high bactericidal effect in vitro, or even in vivo, on one microorganism, as *B. typhosus* or the pneumococcus, will show a similar effect on the microorganisms of other diseases as tuberculosis or anterior poliomyelitis.

THE RÔLE OF BACTERICIDAL TESTS IN VITRO IN BACTERIAL CHEMOTHERAPY

In chemotherapeutic studies on bacterial infections, experiments in vitro may be said to have a positive value in preliminary orientation in the development of leads and the study of new compounds as they are produced. Experimental data at hand tend to show that substances possessing a high bactericidal activity in vitro, and particularly in a menstruum of fresh sterile serum, are more likely to exert an inhibitory example, Morgenroth's drug, optochin (ethylhydrocuprein), and its hydrochlorid exert a very high bactericidal action on the pneumococcus hydrochlorid, exert a very high bactericidal action on the pneumococcus in vitro and are likewise effective to some extent in vivo; other cinchona derivatives, including certain salts of quinin, possessing more or less bactericidal value in vitro are likewise effective to a certain degree in vivo.¹ Arsenobenzol has been found to possess the highest parasitotropic activity on *T. equiperdum* in vitro of a number of substances tested,² and, as well known, this drug exerts the best therapeutic or parasitotropic effects in vivo. Unfortunately, however, other substances that are highly bactericidal in vitro, as the mercurials, also possess a high degree of toxicity for the living animal, and all efforts

¹ Cohen, Kolmer, and Heist, Jour. Infect. Dis., 1917, 20, p. 81.

² Kolmer, Schamberg, and Raiziss, *ibid.*, p. 10.

of the chemotherapeutist have so far failed to lower materially the toxicity of these compounds.

Since the time of the earliest discoveries in bacteriology and of bactericides, substances highly bactericidal in the test tube have been known to fail to exert any appreciable influence *in vivo* when given in safe doses. This lack of effect may be due to the high organotropic or toxic effect of the substance on the body cells in general or on a particular group of cells of vital centers, precluding the use of a bactericidal quantity; to insolubility and difficulty of administration; to rapid union with the proteins of the body and the formation of inert compounds; to rapid elimination; to failure to reach the microorganism in a lesion, and to still other causes. Of these possibilities the first mentioned is of primary importance; but the method and manner of attack of the bactericidal drugs on the protoplasm of cells, aside from coagulation, is almost unknown. Nevertheless, it is possible by systematic modification of the molecule through addition, removal, or substitution of certain atom groups, to produce in some instances a sufficient lowering of toxicity to give an available therapeutic agent. The demonstration of this fact constitutes the great triumph of Ehrlich and opens a fascinating field of research to chemist and biologist.

It is highly probable that experimental studies tending to increase the monotropism of a drug *in vitro* and particularly in a menstruum of serum will prove of value in chemotherapeutic work. For example, ethylhydrocuprein, which shows the highest selective action upon the pneumococcus *in vitro*, likewise proves most bactericidal *in vivo*. Similar facts may be proved in the future in connection with the micro-parasites of tuberculosis and of typhoid fever, and staphylococci, and other infective bacteria.

On the other hand, a substance failing to exert parasitotropic action either *in vitro* or *in vivo* may still prove of value as a basis of composition, and offer a valuable lead in chemotherapeutic research. For example, arsenic in the form of the trioxid has no appreciable effect on *T. equiperdum* *in vivo* and but slight effect *in vitro*,² and yet it forms the basis of arsenobenzol, which is so highly parasitotropic both *in vitro* and *in vivo*.

Likewise, it is possible that a chemical may be more efficacious *in vivo* than *in vitro* by reason of the formation of new and more active compounds *in vivo*; or by exciting the body cells to produce antibodies for the parasitic antigen or chemically facilitating such production; or by the stimulation of phagocytosis, as suggested by some of our experi-

ments in the case of quinin compounds in pneumococcus infections.³ Still, as a general rule, it appears that substances without appreciable effect in vitro are likely to be similarly inert in vivo. Arsenobenzol offers no exception to the rule; contrary to the general impression, it possesses a marked trypanocidal activity in vitro.

It is highly probable, therefore, that experiments in vitro have a definite value in chemotherapeutic research as methods of preliminary orientation and in the development of monotropic chemicals. This value is greater when the identical microparasite causing the definite infection under study is employed in the tests. In the absence of a pure culture of the particular microparasite against which a destroyer is sought, or in the presence of insuperable technical difficulties, other and more easily cultivated organisms closely or remotely related, or even of a different biologic order, may be employed; as in the use of *B. typhosus* and other bacteria by Jacobs and his colleagues⁴ in chemotherapeutic studies concerning anterior poliomyelitis.

SPECIAL REQUISITES OF BACTERICIDAL TESTS IN VITRO IN BACTERIAL CHEMOTHERAPY

It is well known that many factors influence the results of bactericidal tests, and many investigators, notably Rideal and Walker,⁵ and Anderson and McClintic⁶ have sought to establish standard methods for the bacteriologic standardization of disinfectants. Consideration of the physical and chemical laws underlying the action of chemicals on bacteria in vitro are to be found in their papers, and in that of Phelps⁷ and others.

For chemotherapeutic studies 2 main kinds of in-vitro tests may be employed: (1) a simple technic observing the first principles governing these reactions, particularly a uniform temperature, and employing an easily cultivated microorganism, as *B. typhosus*; or (2) a technic aiming at conditions more closely approximating those operative in the living animal.

As a result of a large amount of work with various methods, one fact stands out clear, and that is the necessity of adopting a uniform technic. The most marked variations in bactericidal power appear as the test-methods employed are varied.

³ Kolmer, Steinfeld, and Cohen, *Jour. Infect. Dis.*, 1917, 20, p. 101.

⁴ *Jour. Exper. Med.*, 1916, 23, p. 569.

⁵ *Jour. Roy. San. Inst.*, 1903, 24, p. 424.

⁶ *Jour. Infect. Dis.*, 1911, 8, p. 1.

⁷ *Ibid.*, p. 27.

With certain microorganisms, as *B. typhosus* and staphylococci, either the Hygienic Laboratory, or the Rideal-Walker method may be employed to determine the bactericidal action of new compounds developed in the course of chemotherapeutic studies. But, as we shall point out later, these methods are not adapted to work with microorganisms less hardy and requiring special culture media, as the pneumococcus, gonococcus, and *S. pallidum*. Furthermore, it has appeared to us that for the special purposes of chemotherapeutic study an acceptable method should approach as nearly as possible the conditions presented in the living animal, particularly with reference to temperature.

Here we may summarize what we consider to be the essentials of an acceptable in-vitro bactericidal test for the special purpose of chemotherapeutic studies in bacterial infections. All these factors have been subjected to experiment, using in most instances quinin compounds and various types of pneumococci.

1. The test should be conducted at a temperature of 37 C. instead of 20 C. in order to approach more nearly conditions in the living animal. The influence of temperature on the chemical reaction between bacterium and bactericide has been uniformly emphasized and constitutes a very important factor.

2. The solution of chemical should contain some organic matter, as the protein of broth, sterile inactivated serum, or ascites fluid. Wide variations in bactericidal power are noted according to whether the menstruum is serum, pus, or salt solution.

According to Wright⁸ and his co-workers, Morgenroth's drug has almost the same bactericidal power in vitro in a solution in serum as in salt solution, while the bactericidal activity of other substances is greatly reduced; our own experiments tend to confirm this important observation as to cinchona derivatives in general, and demonstrate the probable value of using serum or ascites fluid in these tests.⁹

When large numbers of new compounds are to be tested at frequent intervals, and particularly in an effort to build up a monobacteriotropic chemical, the simpler technic fulfilling at least the fundamental laws of chemical reaction and utilizing sterile salt solution as the menstruum, may be employed; but when an effort is being made to obtain from in-vitro tests data of greater value in relation to the probable effect of a given parasitotrope in vivo, serum is to be preferred.

3. In the chemotherapeutic study of a definite infection the causative microorganism should be employed whenever possible. For example, ethylhydrocuprein hydrochlorid, quinin salts, and other cinchona derivatives show a highly selective affinity for the protein of the pneumococcus, and in employing in-vitro tests in the chemotherapeutic study of pneumococcus infections, these observations demonstrate the necessity of using the homologous organism.

It would also appear advisable to employ a freshly isolated culture from human or experimental lesions in the conduct of certain tests which aim to

⁸ Lancet, 1912, 11, pp. 1633, 1701.

⁹ Cohen, Kolmer, and Heist, Jour. Infect. Dis., 1917, 20, p. 40.

be conclusive. This has been found especially true of the pneumococcus. The presence or absence of capsules and the degree of virulence of the strain for white mice and rabbits appear to influence markedly the results of tests in vitro. For routine tests, however, it is better to employ a uniform and known culture or cultures well adapted for growth under artificial conditions, but preferably retaining some degree of virulence.

It is necessary to employ in the tests a sufficient number of microorganisms to yield well-defined results. In work with the pneumococcus and similarly less hardy bacteria, appropriate culture media must be employed and frequent counts made by the plating of 24-hour cultures to determine the approximate numbers of living microorganisms present.

ANTISEPTIC AND BACTERICIDAL TESTS

Tests may be conducted after ordinary methods with low concentrations of the chemical over long periods of time, with apparent inhibition of the microorganism which may amount to true killing (the 'antiseptic' test); or with higher concentrations but shorter periods of exposure, and a definite attempt to determine whether or not killing has resulted (the 'bactericidal' test). Plating methods attempt to measure the degree of bactericidal activity after a definite exposure to varying concentrations of the chemical, according to the number of bacteria killed.

In so far as the application of these tests to conditions in the living animal is concerned, it is probable that the antiseptic test is more closely parallel, since in both, the chemical is highly diluted and the exposure prolonged. Furthermore, the number of microorganisms required for definite results can be much less than in the bactericidal test with higher concentration and shorter exposure. Except for accuracy in study, and in maladies in which time may be of high therapeutic importance, there is no real reason for this differentiation between antiseptic and bactericidal properties; not only because they depend on identical reactions, but also because prophylactically and therapeutically a slow rate of killing over a long time may be just as efficacious as a rapid rate in a shorter time.

As pointed out by Phelps⁷ and amply confirmed by our work,⁸ antiseptic power, or true disinfection at low concentrations for long periods, will show a relation between two chemicals quite different from that shown at higher concentrations and shorter periods of exposure. One compound may be a superior bactericide in high concentration and lose rapidly in efficacy with dilution and hence prove

to be a poor antiseptic; while the other, tho of lesser value in high concentration, retains its power better with dilution.

OBJECT OF THIS INVESTIGATION

With these considerations in mind, we have devised or modified a number of different methods with the definite purpose of determining their advantages, disadvantages, and relative values as bactericidal tests in chemotherapeutic studies on bacterial infections, particularly in pneumonia. The results of these tests with a large number of quinin compounds and other substances acting on pneumococci belonging to the serologic Types I, II, and III, are given in a previous paper.⁹

RIDEAL-WALKER AND HYGIENIC LABORATORY METHODS AND MODIFICATIONS OF THESE FOR BACTERICIDAL TESTS WITH PNEUMONIA

While these methods have proved of distinct value in testing the bactericidal value of chemicals against such microorganisms as *B. typhosus* and staphylococci, they have proved, in our experiments, unsuited for work with the pneumococcus. With the addition of 0.1 c.c. of a 24-hour dextrose-broth culture of a pneumococcus to 5 c.c. of varying dilutions of the bactericide in distilled water at 20 C., subculturing with a 4-mm. loop of platinum wire nearly always resulted in sterile controls, or at least gave very irregular results. For this reason we have modified these methods with the object of obtaining uniform results and adapting them to work with the pneumococcus in bacterial chemotherapy, (*a*) by conducting the tests at 37 C. instead of 20 C.; (*b*) by having present some organic matter in the solutions of chemical; (*c*) by using larger amounts of culture in the tests; and (*d*) by employing selective culture media for the seed tubes.

Media.—In all our experiments we have employed the broth found by Cole and his associates to be well adapted for cultivating the pneumococcus, namely, one of fresh beef containing 0.1% dextrose, with an end reaction of +0.2, and sterilized for a minimal length of time. Two drops of sterile, freshly defibrinated human or rabbit blood were added to each tube and all incubated for at least 48 hours to ensure sterility before use. Ten cubic centimeters of the broth were placed in each tube. As these broths vary in their ability to support the pneumococcus cultures, it was found advisable to test out each lot before accepting it for work.

Organism.—Twenty-four-hour broth cultures of stock pneumococci belonging to the first three serologic types were used; but for routine work Type I was finally selected. All cultures were first examined for purity by stained smears and the supernatant cultures used without shaking or filtering, as smears generally showed the cocci to be well scattered. One cubic centimeter

TABLE 1

RESULTS OF BACTERICIDAL TESTS BY MODIFIED RIDEAL-WALKER AND HYGIENIC
LABORATORY METHODS

Name: hydroquinin hydrochlorid (methylhydrocuprein).
Culture used: pneumococcus, Type II, 24-hour broth culture; 0.0001 to 0.0002 c.c. fatal
for white mice in 48 hours.
Temperature of medication: 37 C.
Proportion of culture and chemical: 1 c.c. + 5 c.c.
Organic matter present: protein constituents of the broth.
Subculture media: special blood broth containing 0.1% dextrose.
Reaction: + 0.2.
Quantity in each tube: 10 c.c.

Substance	Dilution	Exposure in Minutes						Phenol Coefficient
		2½	5	7½	10	12½	15	
Phenol.....	1:200	—	—	—	—	—	—	200 <u>5600</u> 40.0
	1:300	+	—	—	—	—	—	
	1:400	+	+	—	+	—	—	
	1:600	+	+	+	—	+	+	400 <u>2400</u> 60
	1:800	+	+	+	+	+	+	2 <u>100</u> 50 =
	1:1200	+	+	+	+	+	+	Coefficient
	1:8000	—	—	—	—	—	—	
	1:12,000	+	—	—	+	—	—	
Hydroquinin.....	1:18,000	+	+	—	—	+	—	
	1:24,000	+	+	+	+	+	—	
	1:30,000	+	+	+	+	+	+	
	1:60,000	+	+	+	+	+	+	
	1:120,000	+	+	+	+	+	+	
Control.....		+	+	+	+	+	+	

— = sterile

+ = growth of pneumococcus

instead of 0.1 c.c. of culture was used, added to 5 c.c. of the solution of chemical.

Temperature.—Tests were conducted at both the standard temperature of 20 C. and at 37 C. in a specially constructed water bath adapted to the purposes of this work. The latter appeared to yield more regular results and higher bactericidal values.

Dilutions.—Dilutions of both the chemical under study and of the control phenol were prepared in the broth mentioned, instead of distilled water. The presence or absence of the few drops of blood did not appear to influence the results materially.

A carefully standardized 5% solution of phenol in distilled water was employed in all tests as a stock dilution, and to equalize matters 5% and 1% stock solutions of the various chemicals in sterile distilled water were first prepared and further dilutions made with the broth.

Seeding.—We have employed both the regulation 4-mm. loop of the Hygienic Laboratory method and graduated pipets (transferring 0.05 c.c.) for purposes

of inoculating the seed tubes. With the pneumococcus, the latter technic yielded more constant results by reason of transferring large numbers of cocci; but it has the disadvantages of transferring a portion of the chemical, and of being more difficult of execution.

Controls.—In each experiment several controls were included by placing 1 c.c. of culture in 5 c.c. of broth at 37° C. and subculturing with sterile pipets at the regular intervals of 2½, 5, 7½, 10, 12½, and 15 minutes.

Incubation.—All subcultures were incubated for 48 hours at 37° C., and then the results read and recorded.

Method of Conducting the Test and Determining the Coefficient.—With the modifications briefly described, the technic was conducted after the standard methods, and the coefficient determined, when possible, after the method of the Lancet Commission, which was adopted into the Hygienic Laboratory method, namely, the mean between the strength and time coefficients.

A fairly typical experiment selected from a number conducted with these modifications is shown in Table 1.

The results, as shown in Table 1, were irregular and generally even poorer than the illustration, despite a considerable number of repetitions by two different workers. The straight Rideal-Walker and Hygienic Laboratory methods were entirely unsatisfactory in work with the pneumococcus and the modified method described, while yielding better results, was found inferior to other methods to be detailed later. With *B. typhosus* as the test organism, however, these methods have proved of value in our experience in testing the bactericidal activity of new compounds, during the course of other studies in bacterial chemotherapy. A further modification consisting of plating instead of seeding the broth tubes shows when there is partial killing and diminution in the numbers of bacilli.

CENTRIFUGE METHOD

Bactericidal tests were also conducted with what may be called a centrifuge method, in which the bactericide is permitted to act on the pneumococci for a certain length of time, after which the microorganisms are removed for culture.

One cubic centimeter of varying dilutions of the test substance in sterile broth, was placed in each of a series of sterile test tubes suitable for centrifugation at high speed and furnished with sterile rubber stoppers. To each of these tubes and to each of several controls (1 c.c. of broth alone) was carefully added 1 c.c. of a 24-hour broth culture of a pneumococcus, and the whole gently mixed and incubated for 1 hour. At the end of this time from 6 to 8 c.c. of sterile broth were added to each tube, and the contents centrifugated at high speed for from 15 to 20 minutes. The supernatant fluid was then removed under sterile conditions, 6 to 8 c.c. of sterile broth added, and after the mass of cocci had been stirred up with a platinum wire, the

whole was centrifugated for a similar period in order to remove all traces of the chemical. After the washing, fresh sterile broth was added to each tube and this culture incubated for 48 hours, after which the results were read.

The results of experiments with 3 different quinin compounds are shown in Table 2.

TABLE 2

RESULTS OF BACTERICIDAL TESTS BY THE CENTRIFUGE METHOD WITH PNEUMOCOCCUS TYPE I
(EXPOSURE OF 1 HOUR)

Test	24-hr. Broth Culture	Final Dilution	Results		
			Quinin and Urea Hydrochlorid	Ethyhydrocuprein Hydrochlorid	Hydroquinin Hydrochlorid
1	1 c.c.	1:100	—	—	—
2		1:500	+	—	+
3		1:300	+	—	+
4		1:400	+	—	+
5		1:500	+	—	+
6		1:600	+	—	+
7		1:700	+	—	+
8		1:800	+	—	+
9		1:900	+	+	+
10		1:1000	+	+	+
11		Control	+	+	+

— = sterile
+ = growth

This method consumes considerable time and requires extreme care to prevent contamination, but its results, as a rule, are sharp and definite. By varying the interval of exposure, information may be gained as to the rapidity of bactericidal action.

As shown in the table, ethylhydrocuprein hydrochlorid was 8 times more bactericidal than quinin and urea hydrochlorid and hydroquinin hydrochlorid.

PIPET METHOD

For comparison of the bactericidal activity of quinin compounds in solutions of sera and in normal salt solution, we have found the following method useful, because of the small amounts of serum required for a large number of tests.

Both ordinary capillary and looped pipets (Wright) prepared of ordinary 7-mm. glass tubing were used. The mouth end of each was plugged with cotton and all sterilized by dry heat before use.

Varying dilutions of the compound were prepared in sterile normal human or horse serum in quantities of 1 c.c., in a series of small sterile tubules held in a slanting position in plasticine and inoculated with 0.2 c.c. of a 24-hour broth culture of a pneumococcus. (These sera alone were without appreciable bactericidal effect on the pneumococci.)

At intervals of 5, 10, 15, 20, and 30 minutes, each dilution was subcultured by drawing a small portion of each, averaging about 5 c.mm., into the

pipet, the bulb of which had been filled just before with a special pneumococcus blood dextrose broth.

Each pipet was sealed in a peep flame immediately after filling and incubated for 48 hours, after which the results were read.

In each experiment a set of controls, prepared by adding 0.2 c.c. of culture to 1 c.c. of serum or salt solution respectively and subculturing at the same intervals, was included.

Tables 3, 4, and 5 show the results observed with this method with dilutions of ethylhydrocuprein hydrochlorid in sterile human serum acting on stock cultures of pneumococci belonging to Types I, II, and III.

The results obtained with this method were clear cut and sharp, and it possesses the distinct advantage of affording a practical and economical means of determining the bactericidal activity of substances in a menstruum of either serum, ascites fluid, salt solution, or distilled water.

With this method we have tested the bactericidal activity of quinin compounds and other disinfectants in solution in serum and salt solution on pneumococci of various types, the results being summarized elsewhere.⁹

THE COMBINED IN-VITRO-VIVO METHOD

In this method 1 c.c. of a dilution of the bactericide in serum, broth, or salt solution is allowed to act on 1 c.c. of a culture of pneumococci of known virulence, so diluted that 0.1 c.c. contains 100 minimal lethal doses, at a temperature of 37 C. and at intervals of 5, 15, 30, 45, and 60 minutes; 0.2 c.c. of the mixture is injected intraperitoneally into mice to determine when killing of the pneumococci has occurred.

The technic of this test is as follows:

1. A 24-hour broth culture of a pneumococcus is titrated by intraperitoneal injection into mice to determine the M. L. D. in a period of 24-36 hours after injection. This culture is then diluted with sterile broth until 100 times the M. L. D. is contained in each 0.1 c.c.
2. A dilution of the bactericide in serum, broth, or salt solution is prepared—for example, 1:1000 ethylhydrocuprein hydrochlorid—and 1 c.c. placed in a small sterile tubule in a water bath at 37 C. After a few minutes 1 c.c. of the diluted culture is added and the contents gently mixed. The dilution of bactericide has now been doubled (1:2000).
3. At intervals of 5, 15, 30, 45, and 60 minutes, 0.2 c.c. of the mixture is injected into the peritoneal cavity of a white mouse. The 1-c.c. graduated syringe used for this purpose is sterilized before each injection.
4. One or more controls are included by mixing 1 c.c. of the diluted culture with 1 c.c. of the same fluid used in preparing the dilution of disinfectant (serum, broth, or salt solution); the tubule or tubules are kept in the water bath and 0.2 c.c. injected into a series of mice at the same intervals.

TABLE 3

RESULTS OF BACTERICIDAL TESTS BY THE PIPET METHOD (GERMICIDAL ACTION OF ETHYL-HYDROCUPREIN IN SERUM UPON PNEUMOCOCCUS TYPE I)

Test	Dilutions	Results (min.)				
		5	10	15	20	30
1	1:1000	—	—	—	—	—
2	1:2000	—	—	—	—	—
3	1:10,000	—	—	—	—	—
4	1:20,000	—	—	—	—	—
5	1:100,000	+	—	—	—	—
6	1:200,000	+	+	+	+	+
7	1:500,000	+	+	—	+	+
8	1:800,000	+	+	+	+	+
9	1:1,000,000	+	+	+	+	+
10	Control	+	+	+	+	+

— = sterile
+ = growth

TABLE 4

RESULTS OF BACTERICIDAL TESTS BY THE PIPET METHOD (GERMICIDAL ACTION OF ETHYL-HYDROCUPREIN IN SERUM UPON PNEUMOCOCCUS TYPE II)

Test	Dilutions	Results (min.)				
		5	10	15	20	30
1	1:1000	—	—	—	—	—
2	1:2000	—	—	—	—	—
3	1:10,000	—	—	—	—	—
4	1:20,000	+	+	+	+	+
5	1:100,000	+	+	+	+	+
6	1:200,000	+	+	+	+	+
7	1:500,000	+	+	+	+	+
8	1:800,000	+	+	+	+	+
9	1:1,000,000	+	+	+	+	+
10	Control	+	+	+	+	+

— = sterile
+ = growth

TABLE 5

RESULTS OF BACTERICIDAL TESTS BY THE PIPET METHOD (GERMICIDAL ACTION OF ETHYL-HYDROCUPREIN IN SERUM UPON PNEUMOCOCCUS TYPE III)

Test	Dilutions	Results (min.)				
		5	10	15	20	30
1	1:1000	—	—	—	—	—
2	1:2000	—	—	—	—	—
3	1:10,000	—	—	—	—	—
4	1:20,000	—	—	—	—	—
5	1:100,000	+	+	—	—	—
6	1:200,000	+	+	+	+	—
7	1:500,000	+	+	+	+	—
8	1:800,000	+	+	+	+	+
9	1:1,000,000	+	+	+	+	+
10	Control	+	+	+	+	+

— = sterile
+ = growth

5. The mice are kept under observation for at least 6 days, and at autopsy the blood of the heart is examined by smear and culture for pneumococci. During the first 48 hours death is generally due to pneumococci; after this time the blood is usually found sterile and death is ascribed to the toxicity of the drug injected.

The results of a test conducted in this manner are shown in Table 6.

TABLE 6
RESULTS OF BACTERICIDAL TESTS BY THE COMBINED IN-VITRO-VIVO METHOD

Culture: 24-hour broth culture of pneumococcus, Type I; 0.00001 c.c. fatal by intraperitoneal injection in 48 hours; diluted with broth so that 0.1 c.c. contained 100 M.L.D.
Substance: quinin bisulfate; dilution 1:100 in sterile salt solution.
Proportion of bactericide and culture: 1 c.c. + 1 c.c. diluted culture (final dilution of bactericide then acting on the cocci was 1:200).

Test	Exposure (min.)	Result (days)						Remarks
		1	2	3	4	5	6	
1	5		Died					Pneumococci in blood
2	15						Living	
3	15						Living	
3	30						Living	
4	45						Living	
5	60						Living	
6	Control	Died						

The results observed with this method in testing the bactericidal activity of a number of quinin compounds and derivatives were comparatively definite. With different cultures of the same serologic type of pneumococcus, however, slightly varying results were observed, so that as usual in such animal experiments it is advisable to conduct an experiment in duplicate in order that true results may be obtained.

The method has the distinct advantage of controlling the result by animal inoculation, which in the case of a highly virulent culture of a pneumococcus constitutes in our experience a delicate test for viable microorganisms. The disadvantage of the test lies in the fact that in such a combined in-vitro-vivo method, some of the drug is injected intraperitoneally and probably continues its bactericidal activity within the peritoneal cavity for a short time at least. For example, in the experiment detailed, 0.001 gm. of quinin bisulfate was injected into each of the mice (averaging about 15 gm. in weight), corresponding to about 0.06 gm. per kilogram of body weight.

ANTISEPTIC METHOD

This method, which is an adaptation of older methods, is called an 'antiseptic' test, because it consists in using high dilutions of the disinfectant against small numbers of the microorganism over a long period of time.

This method has proved in our hands one of the best because of the sharpness of its results, the ease of its manipulation, and its adaptability for testing substances which are practically insoluble in water, salt solution, or broth.¹⁰

It is conducted as follows:

1. In a series of 10 sterile test tubes are prepared dilutions of the disinfectant in 1 c.c. of sterile broth or normal salt solution, which are 10 times higher than the final dilutions desired.

2. To each tube are added 9 c.c. of sterile broth (in pneumococcus work it is not necessary to use blood in the broth); to the control tube are added 1 c.c. of the same fluid used in preparing the dilutions of disinfectant and 9 c.c. of broth.

3. All tubes are now sterilized in the Arnold sterilizer for one-half hour and incubated over night to test for sterility of the disinfectant and broth and to bring the temperature to 37 C. (In the case of quinin and mercurial compounds the disinfectant values are not impaired by the process of sterilization; with volatile disinfectants it is better practice to omit sterilization, but not the preliminary incubation.)

4. On the following day each tube is seeded with a drop of pneumococcus culture (about 0.05 c.c.) and gently mixed. This does not materially alter the dilution.

5. All tubes are incubated at 37 C. for at least 8 days. As growths appear in the tubes, these are tested for purity and to guard against accidental contamination, by means of stained smears.

6. The results are read and recorded by daily inspection of the tubes. The controls invariably show a good growth after 24 hours' incubation. Perfectly clear tubes are recorded as sterile. On successive days lower and lower dilutions may show a growth of pneumococci up to the 6th day, after which the results remain stationary over a long period of time. The highest values of the bactericide are found on the first day; after this time inhibition may be overcome in one or more of the dilutions and growths occur. In the case of *B. typhosus* and staphylococci, growths may appear as late as the 7th day, but very seldom thereafter. We have recorded the bactericidal value of a substance according to the highest dilution remaining sterile on the 8th day.

In so far as the pneumococcus is concerned, tests of the clear or sterile tubes after 8 days by subculture in blood agar and by intraperitoneal injection into mice and intravenous injection into rabbits, are practically always negative, thus indicating that the microorganisms have been 'completely killed.'

The results of a few experiments with pneumococci of various types and various quinin derivatives are shown in Tables 7 to 12.

The results observed with a large number of substances as tested by this method are given elsewhere;⁹ here it may be stated that on account of the uniformity of results as determined by different workers using the technic with the same set of substances, the sharpness of the results, the ease of manipulation and possibility of detecting finer

¹⁰ Schamberg and Kolmer, *Jour. Am. Med. Assn.*, 1914, 62, p. 1950.

TABLE 7

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING ETHYLHYDROCUPREIN
HYDROCHLORID AND PNEUMOCOCCUS TYPE I

Test	Culture	Broth	Final Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:16,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:50,000	—	—	—	—	—	—	—	—
7			1:80,000	—	—	—	—	—	—	—	—
8			1:100,000	—	—	—	—	—	—	—	—
9			1:200,000	—	—	—	—	—	—	—	—
10			1:800,000	—	—	—	—	—	—	—	—
11			1:1,000,000	—	—	—	—	—	—	—	—
12			1:1,500,000	—	—	—	—	—	—	—	—
13			1:2,000,000	—	—	+	+	+	+	+	+
14			1:3,000,000	—	—	+	+	+	+	+	+
15			1:4,000,000	—	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile

+ = growth

TABLE 8

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING ETHYLHYDROCUPREIN
HYDROCHLORID AND PNEUMOCOCCUS TYPE II

Test	Culture	Broth	Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:16,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:50,000	—	—	—	—	—	—	—	—
7			1:80,000	—	—	—	—	—	—	—	—
8			1:100,000	—	—	—	—	—	—	—	—
9			1:200,000	—	—	—	—	—	—	—	—
10			1:800,000	—	—	—	—	—	—	—	—
11			1:1,000,000	—	—	—	—	—	—	—	—
12			1:1,500,000	—	—	—	—	—	—	—	—
13			1:2,000,000	—	—	—	—	—	—	—	—
14			1:3,000,000	—	—	—	—	—	—	—	—
15			1:4,000,000	—	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile

+ = growth

TABLE 9

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING ETHYLHYDROCUPREIN HYDROCHLORID AND PNEUMOCOCCUS TYPE III

Test	Culture	Broth	Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:16,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:50,000	—	—	—	—	—	—	—	—
7			1:80,000	—	—	—	—	—	—	—	—
8			1:100,000	—	—	—	—	—	—	—	—
9			1:200,000	—	—	—	—	—	—	—	—
10			1:800,000	—	—	—	—	—	—	—	—
11			1:1,000,000	—	—	—	—	—	—	—	—
12			1:1,500,000	—	—	—	—	—	—	—	—
13			1:2,000,000	—	—	+	+	+	+	+	+
14			1:3,000,000	—	+	+	+	+	+	+	+
15			1:4,000,000	+	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

TABLE 10

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING QUININ BISULFATE AND PNEUMOCOCCUS TYPE I

Test	Culture	Broth	Final Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:16,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:50,000	—	—	—	—	—	—	—	—
7			1:80,000	—	—	—	—	—	—	—	—
8			1:100,000	—	—	—	—	—	—	+	+
9			1:200,000	+	+	+	+	+	+	+	+
10			1:800,000	+	+	+	+	+	+	+	+
11			1:1,000,000	+	+	+	+	+	+	+	+
12			1:1,500,000	+	+	+	+	+	+	+	+
13			1:2,000,000	+	+	+	+	+	+	+	+
14			1:3,000,000	+	+	+	+	+	+	+	+
15			1:4,000,000	+	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

TABLE 11

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING QUININ AND UREA
HYDROCHLORID AND PNEUMOCOCCUS TYPE I

Test	Culture	Broth	Final Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:1000	—	—	—	—	—	—	—	—
2			1:2500	—	—	—	—	—	—	—	—
3			1:5000	—	—	—	—	—	—	—	—
4			1:10,000	—	—	—	—	—	—	—	—
5			1:50,000	—	+	+	+	+	+	+	+
6			1:100,000	+	+	+	+	+	+	+	+
7			1:250,000	+	+	+	+	+	+	+	+
8			1:500,000	+	+	+	+	+	+	+	+
9			1:750,000	+	+	+	+	+	+	+	+
10			1:1,000,000	+	+	+	+	+	+	+	+
11			1:2,000,000	+	+	+	+	+	+	+	+
12			1:3,000,000	+	+	+	+	+	+	+	+
13			1:4,000,000	+	+	+	+	+	+	+	+
14			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

TABLE 12

RESULTS OF BACTERICIDAL TESTS BY THE ANTISEPTIC METHOD USING HYDROQUININ
HYDROCHLORID AND PNEUMOCOCCUS TYPE I

Test	Culture	Broth	Final Dilutions	Results (days)							
				1	2	3	4	5	6	7	8
1	0.05 c.c.	10 c.c.	1:5000	—	—	—	—	—	—	—	—
2			1:8000	—	—	—	—	—	—	—	—
3			1:10,000	—	—	—	—	—	—	—	—
4			1:15,000	—	—	—	—	—	—	—	—
5			1:20,000	—	—	—	—	—	—	—	—
6			1:30,000	—	—	—	—	—	—	—	—
7			1:40,000	—	—	—	—	—	—	—	—
8			1:50,000	—	—	—	—	—	—	—	—
9			1:80,000	—	+	+	+	+	+	+	+
10			1:100,000	—	+	+	+	+	+	+	+
11			1:200,000	+	+	+	+	+	+	+	+
12			1:500,000	+	+	+	+	+	+	+	+
13			1:1,000,000	+	+	+	+	+	+	+	+
14			1:2,000,000	+	+	+	+	+	+	+	+
15			1:3,000,000	+	+	+	+	+	+	+	+
16			Control	+	+	+	+	+	+	+	+

— = sterile
+ = growth

grades and degrees of antiseptic and bactericidal power, we can recommend the method as a simple one for testing new compounds both soluble and insoluble, particularly the latter, in the course of chemotherapeutic or other studies.

PLATING METHOD

This bactericidal test endeavors to determine the dilution of drug producing death of some considerable part or all of the test microorganisms (partial or total germicidal action) in a given period of time, by removing a very small amount of the mixture of disinfectant and culture, and plating with sufficient medium to practically dilute out of action the small quantity of disinfectant carried over.

TABLE 13

RESULTS OF BACTERICIDAL TESTS BY THE PLATE METHOD

Substance: ethylhydrocuprein hydrochlorid.

Culture: 24-hour broth culture of pneumococcus, Type I.

Test	Dilutions	Results in 48 Hours	
		Plates	Test Tubes
1	1 : 10,000	Sterile	Sterile
2	1 : 20,000	Sterile	Sterile
3	1 : 40,000	Sterile	Sterile
4	1 : 80,000	Inhibition	Growth
5	1 : 100,000	Inhibition	Growth
6	1 : 200,000	Inhibition	Growth
7	1 : 400,000	Inhibition	Growth
8	1 : 1,000,000	Inhibition	Growth
9	1 : 2,000,000	Growth	Growth
10	1 : 4,000,000	Growth	Growth
11	Control	Growth	Growth

The test is conducted as follows:

1. A series of dilutions of the disinfectant in serum, ascites fluid, broth, or normal salt solution in amounts of 1 c.c. is prepared in sterile test tubes of sufficient size to hold 10 c.c. A stock dilution of the disinfectant in distilled water or salt solution is prepared and sterilized beforehand.

2. These tubes are incubated at 37 C. for 24 hours to determine sterility and to bring the temperature to 37 C.

3. To each tube is now added 1 c.c. of a 1:10 dilution of a 24-hour broth culture of the test microorganism; the final dilution of disinfectant acting on the microorganisms is thereby doubled.

4. Controls are prepared in the same manner, the disinfectant being omitted.

5. All tubes are now incubated for 24 hours; then 0.05 c.c. or a 4-mm. loopful, of each dilution and control is transferred to sterile petri dishes and cultured with from 8 to 10 c.c. of blood agar-agar (at 40 C.).

6. To the test tubes are now added 8 c.c. of broth.

7. Plates and test tubes are incubated 48 hours, and thereafter examined and the results recorded.

8. The test-tube cultures act as checks on the plates in determining the dilution in which there is complete killing.

The results of an experiment with this technic are shown in Table 13.

In this particular experiment a dilution of 1:40,000 proved bactericidal and one of 1:1,000,000 proved slightly antiseptic; cultivating the plates and test tubes for a longer time shows a variation in results, particularly with reference to the inhibitory, or antiseptic, dose.

With blood agar as the plating medium, we have not found it possible to make accurate counts of the plates; likewise the sediment which may be present in ascites fluid obscures colonies and renders counts inaccurate. All tests have been done in duplicate or in triplicate and the results recorded as they compared with the controls.

SUMMARY

In-vitro bactericidal tests are probably of value in chemotherapeutic studies as based on the general observation that substances most parasitocidal in vitro also show this quality in marked degree in vivo.

In-vitro tests are of value especially for preliminary trials and orientation and as a delicate and trustworthy means of detecting increasing parasitotropism in building up a monotropic chemical.

In-vitro tests should be conducted when possible with the micro-parasite causing the malady under study, as the object of chemotherapy should be the production not only of polybacteriotropic and polyprotozootropic chemicals, but also of monotropic substances for a definite microparasite. In the absence of a pure culture of the particular microparasite under study, a closely allied species may be used.

In conducting in-vitro tests in chemotherapeutic studies 2 main methods may be used: (1) a simple technic fulfilling the primary laws governing these chemical reactions, and (2) a test embracing as many of the factors believed to be operative in vivo, as possible.

It is highly important to adopt a definite technic and adhere to it in every detail in conducting these tests, because different methods yield varying results, depending mainly on whether the substance excels as a bactericide (killing quickly in high concentration, but losing rapidly in bactericidal power in low concentration) or as an antiseptic (retaining bactericidal power to a better extent in low concentration).

As a routine test characterized by simplicity and sharpness of results we have found the antiseptic test, or tube method, as described, to be of particular value. For detecting degrees of bactericidal and antiseptic activity, the plate method is quite satisfactory. For conducting tests in a menstruum of serum, the pipet method and the combined in-vitro-vivo method are to be recommended.

THE PROTECTIVE AND CURATIVE VALUE OF QUININ
AND UREA HYDROCHLORID, ETHYLHYDROCU-
PREIN, AND OTHER CINCHONA DERIVA-
TIVES IN EXPERIMENTAL PNEU-
MOCOCCUS INFECTIONS *

STUDIES IN PNEUMONIA, III

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In previous communications^{1, 2} we have summarized the results of our studies, with various methods, on the bactericidal action of various cinchonics on pneumococci in vitro, showing that these substances as a group, and ethylhydrocuprein in particular, exert a specific and relatively high bactericidal action on type strains of this microorganism. The object of the present paper is to present briefly the results of our studies concerning the toxicity of these compounds for animals and their protective and curative influence with respect to experimental pneumococcus infections produced by the intraperitoneal and intravenous injection of highly virulent cultures.

The empirical use of cinchona and of quinin compounds in the treatment of acute pneumonia is old, and the power of these agents, when given in sufficient dose sufficiently early, to arrest the progress of pneumonic toxemia, even tho the accompanying tissue changes may not be materially affected, is attested by numerous observers whose conclusions have indeed been disputed, but whose general competency as diagnosticians and pathologists cannot be questioned. In a previous paper¹ we have cited some of these observations.

Influenced less by this tradition than by the effects of the use of quinin compounds as observed in experimental trypanosomiasis, and by the fact that certain peculiar biologic relationships (as bile solubility) have been found by Schilling³ and Neufeld^{4, 5} to exist between trypanosomes and spirilla on the one hand and pneumococci on the other, Morgenroth and Levy⁶ chose quinin and its derivatives as a basis and lead for chemotherapeutic studies on experimental

* Received for publication November 27, 1916.

¹ Cohen, Kolmer, and Heist, Jour. Infect. Dis., 1917, 20, p. 40.

² Kolmer, Cohen, and Heist, *ibid.*, p. 81.

³ Centralbl. f. Bakteriol., I, O., 1902, 31, p. 452.

⁴ Arb. a. d. k. Gsndhtsamte, 1907, 25, p. 494.

⁵ Ztschr. f. Hyg. u. Infektionskr., 1900, 34, p. 454.

⁶ Berl. klin. Wehnschr., 1911, 48, pp. 1560, 1979.

pneumococcus infections. Best results were observed with optochin (ethylhydrocuprein) a synthetic derivative of hydroquinin (methylhydrocuprein) which latter exists in the cinchona bark or can be prepared synthetically.* In experimental infections of mice the new drug (optochin) was found to prevent the development of infection in some 90% of animals in which treatment was postponed till after inoculation. Later Guttman,⁷ Morgenroth and Kaufman,⁸ and Levy⁹ reported further experiments indicating the protective and curative action of ethylhydrocuprein (base) and its hydrochlorid, in experimental infections in mice. Wright¹⁰ found that the administration of ethylhydrocuprein to man resulted in an increased bactericidal power of the blood. These observations were confirmed by Moore¹¹ with animals. Moore¹² also reported that ethylhydrocuprein exerts a well-marked protective action against experimental pneumococcal infection in mice in the case of type strains of all four groups of pneumococci; this protective action was found efficient against many multiples of the minimal lethal dose. Rosenthal¹³ gives an excellent review of the literature bearing on the chemotherapeutic treatment of pneumonia, leading up to and including the work of Morgenroth and Levy and others.

Clinically, the local application of a 1-2% solution of ethylhydrocuprein hydrochlorid has been reported by a number of ophthalmologists as efficacious in the treatment of pneumococcus ulcers of the conjunctiva and cornea tho somewhat irritating. In the treatment of lobar pneumonia the consensus of opinion would seem to indicate that the drug is not of proved value and particularly must care be exercised in the dosage to avoid the amblyopia which has been reported by a number of investigators. The general statement is made that there is but "a small interval between the therapeutic and the toxic dose."

Smith and Fantus¹⁴ have reported that the minimal lethal dose of ethylhydrocuprein for mice is 0.5 mg. per gram of body weight; the dose for quinin is given as slightly higher or 0.7 mg. per gram of body weight. They found that optochin will reduce blood pressure more than quinin and cause quicker cardiac death. Hunt¹⁵ found the lethal dose of hydroquinin to be from 0.35 to 0.33 mg. per gram, and this was confirmed by Morgenroth and Halberstädter.¹⁶

Our own results follow.

THE LETHAL DOSE

The smallest immediately lethal doses of ethylhydrocuprein hydrochlorid and 3 quinin salts were determined for mice and rabbits by intravenous and intramuscular injection.

* The chemical relationship of the cuprein group to the quinin group is indicated by the following formulas: Cuprein, $C_{19}H_{20}N_2 \cdot OH \cdot OH$; Quinin, $C_{19}H_{20}N_2 \cdot OH \cdot OCH_3$; Hydrocuprein, $C_{19}H_{22}N_2 \cdot OH \cdot OH$; Methylhydrocuprein (Hydroquinin), $C_{19}H_{22}N_2 \cdot OH \cdot OCH_3$; Ethyl Hydrocuprein, $C_{19}H_{22}NOH \cdot OC_2H_5$; Quinethylin (according to Grimaux and Arnaud), $C_{19}H_{22}N_2 \cdot OH \cdot OC_2H_5$.

⁷ Ztschr. f. Immunitätsf., 1912, 15, p. 625.

⁸ Centralbl. f. Bakteriöl., R., 1912, 54, p. 69.

⁹ Berl. klin. Wchnschr., 1912, 49, p. 2486.

¹⁰ On Pharmacotherapy and Preventive Inoculation Applied to Pneumonia in the African Native. 1915. (This little book gives a complete account of the investigations of the author and his associates with ethylhydrocuprein and vaccines in human pneumococcus infections).

¹¹ Jour. Exper. Med., 1915, 22, p. 551.

¹² Ibid., p. 269.

¹³ Ztschr. f. Chemotherap., R., 1912, 1, p. 1149.

¹⁴ Jour. Pharmacol. and Exper. Therap., 1916, 8, p. 53.

¹⁵ Arch. internat. de Pharmacod. et de Thérap., 1904, 12, p. 503.

¹⁶ Sitzungsberichte der Königl. Preuss. Akademie der Wissenschaften (Physikal.-mathemat. Classe), 1911, 1, p. 34.

Lethal Dose by Intravenous Injection.—The results of a series of experiments are summarized in Table 1.

All the drugs were dissolved in sterile distilled water and filtered before injection. The total amount of fluid injected did not exceed 1 c.c. for mice or 5 c.c. for rabbits, and in all instances the rate of injection was such as to require about 5 seconds per cubic centimeter. More rapid injection of ethylhydrocuprein tended to increase its toxic effects. Mice were injected by means of a vein in the tail and rabbits by a marginal vein of the ear.

In mice the effects were first evidenced by attempts to switch the tail, muscular twitchings in all extremities, and death after a brief convulsion. Rabbits either develop spasmodic movements of the hind legs, and then of the forelegs, followed by weakness and complete paralysis of the limbs, dyspnea, collapse, and death, or at once develop a severe convulsion with exophthalmos, and die in a few seconds.

TABLE 1

LETHAL DOSE OF ETHYLHYDROCUPREIN AND OTHER CINCHONA DERIVATIVES BY INTRAVENOUS INJECTION

Substance	Animal	Lethal Dose		Estimated Dose per 60 Kilos (125 lb.)
		Per Gram	Per Kilo	
Ethylhydrocuprein hydrochlorid.....	Mouse.....	.00006	.06	3.6
	Rabbit....	.00006	.06	3.6
Quinin and urea hydrochlorid.....	Mouse.....	.00006	.06	3.6
	Rabbit....	.00005	.05	3.0
Hydroquinin hydrochlorid.....	Mouse.....	.00004	.04	2.4
	Rabbit....	.00005	.05	3.0
Quinin hydrochlorid.....	Mouse.....	.00006	.06	3.6
	Rabbit....	.00005	.05	3.0

The immediately lethal doses given in the table represent the average results observed with mice weighing from 14 to 22 gm. and rabbits weighing from 1200 to 2000 gm. In all instances each dose was given strictly according to body weight. All animals were kept in the laboratory at a temperature varying from 67 to 76 F. Individual animals exhibited varying degrees of resistance; as in all biologic experiments, mathematically accurate and constant results according to dosage per body weight are not possible.

Summary.—The immediate lethal dose of ethylhydrocuprein hydrochlorid was 0.06 gm. and of the other cinchonics, from 0.04 to 0.06 gm., per kilo of body weight. In other words, the toxicity of ethylhydrocuprein hydrochlorid was equal to or slightly greater than that of the other compounds tested. Hydroquinin (the parent of ethylhydrocuprein) appeared slightly more toxic than the other compounds—a result not in accord with the majority of reported observations.

As a general rule, mice withstood slightly larger doses than rabbits. Individual experiments also indicated that young rabbits were more resistant than older and heavier animals, confirming the observations of Moore¹¹ in this respect.

Lethal Dose by Intramuscular Injection.—The results of a series of experiments are shown in Table 2. The results were more irregular than observed with the intravenous route; the doses given are those which proved lethal in the majority of tests.

TABLE 2
LETHAL DOSE OF ETHYLHYDROCUPREIN AND OTHER CINCHONA DERIVATIVES BY SINGLE
INTRAMUSCULAR INJECTION

Substance	Animal	Lethal Dose		Estimated Dose per 60 Kilos (125 lb.)
		Per Gram	Per Kilo	
Ethylhydrocuprein hydrochlorid.....	Mouse....	.0006	.6	36.0
	Rabbit....	.0005	.5	30.0
Quinin and urea hydrochlorid.....	Mouse....	.0005	.5	30.0
	Rabbit....	.0005	.5	30.0
Hydroquinin hydrochlorid.....	Mouse....	.00016– .00025	.16–.25	12.3
Quinin hydrochlorid.....	Mouse....	.0005	.5	30.0
	Rabbit....	.0005	.5	30.0

Mice were injected in the muscles of the thigh; rabbits in the erector spinæ and thigh muscles (usually the latter).

Summary.—The lethal doses of these compounds by intramuscular injection were in general about 10 times larger than the lethal doses by intravenous injection.

Mice withstood slightly larger doses of ethylhydrocuprein hydrochlorid than rabbits.

The toxicity of ethylhydrocuprein hydrochlorid was generally slightly greater than that of the quinin compounds, except in the case of hydroquinin, which proved more toxic for mice than the other compounds.

THE TOLERATED DOSE

The largest tolerated dose of ethylhydrocuprein hydrochlorid and of 3 other cinchona derivatives was determined in a series of experiments on mice and rabbits by intravenous, intramuscular, and subcutaneous injection.

Moore¹¹ has reported that rabbits weighing approximately 2000 gm.

tolerate a single dose of 0.1 gm. of ethylhydrocuprein hydrochlorid subcutaneously and 0.02 to 0.05 gm. per kilo of body weight, when it is given slowly, intravenously.

All drugs were dissolved in sterile distilled water and the technic was the same as that described. All animals were observed for a period of 10 days following the injections. After this time deaths occurred at irregular intervals and it was frequently impossible to determine the exact cause. For the sake of definiteness, therefore, we have given the results as observed after this relatively short interval.

In the tables the smaller and larger doses as found in different experiments, are given; the estimated dose per 60 kilos is based on an average of these.

The results of experiments are summarized in Tables 3, 4, and 5.

Summary.—Ethylhydrocuprein hydrochlorid was tolerated in about the same dosage as the quinin compounds or was slightly more toxic.

As a general rule, mice withstood slightly larger doses than rabbits.

The tolerated intramuscular dose of these compounds was about one-half greater than the intravenous dose.

The tolerated subcutaneous dose was about 4 times greater than the intravenous dose, and from 2 to 3 times greater than the intramuscular dose.

PROTECTIVE VALUE

Experiments on the protective value of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchonics were conducted by administering the drugs first, and then inoculating the animals by intraperitoneal or intravenous injection of multiples of the minimal lethal doses of 24-hour cultures.

Strains of pneumococci belonging to Types I, II, and III were employed; most of our work was conducted with Type I. The minimal lethal doses of these strains were determined by frequent intraperitoneal injection into mice and intravenous injection into rabbits and within 2 days of the time selected for the experiments. Their virulence for mice of about 20 gm. in weight and rabbits of about 1600 gm. was generally at or near the following amounts of 24-hour blood-dextrose-broth cultures:

Type I	{	(mice)	48 hours: 0.000 001 c.c.
		(rabbits)	72 hours: 0.001 to 0.01 c.c.
Type II	{	(mice)	48 hours: 0.000001 c.c.
		(rabbits)	72 hours: 0.002 to 0.02 c.c.
Type III	{	(mice)	48 hours: 0.001 c.c.
		(rabbits)	72 hours: 0.1 to 0.2 c.c.

According to body weight, mice were considerably more susceptible to all strains than rabbits.

TABLE 3

THE TOLERATED DOSE OF ETHYLHYDROCUPREIN AND OTHER CINCHONA DERIVATIVES BY INTRAVENOUS INJECTIONS

Substance	Animal	Tolerated Dose		Estimated Dose per 60 Kilos (125 lb.)
		Per Gram	Per Kilo	
Ethylhydrocuprein hydrochlorid.....	Mouse.....	.00002-.00004	.02-.04	1.8
	Rabbit....	.00002-.00003	.02-.03	1.5
Quinin and urea hydrochlorid.....	Mouse.....	.00002-.00007	.02-.07	2.7
	Rabbit....	.00002-.00005	.02-.05	2.4
Hydroquinin hydrochlorid.....	Mouse.....	.00002-.00006	.02-.06	2.4
	Rabbit....	.00002-.00004	.02-.04	1.8
Quinin hydrochlorid.....	Mouse.....	.00002-.00005	.02-.05	2.1
	Rabbit....	.00002-.00003	.02-.03	1.5

TABLE 4

THE TOLERATED DOSE OF ETHYLHYDROCUPREIN AND OTHER CINCHONA DERIVATIVES BY SINGLE INTRAMUSCULAR INJECTION

Substance	Animal	The Tolerated Dose		Estimated Dose per 60 Kilos (125 lb.)
		Per Gram	Per Kilo	
Ethylhydrocuprein hydrochlorid.....	Mouse.....	.00003 -.00005	.03 -.05	2.4
	Rabbit....	.00003 -.00007	.03 -.07	3.0
Quinin and urea hydrochlorid.....	Mouse.....	.00003 -.00007	.03 -.07	3.0
	Rabbit....	.00003 -.00008	.03 -.08	3.3
Hydroquinin hydrochlorid.....	Mouse.....	.000013-.00004	.013-.04	1.6
	Rabbit....	.00002 -.00006	.02 -.06	2.4
Quinin hydrochlorid.....	Mouse.....	.00003 -.00006	.03 -.06	2.7
	Rabbit....	.00003 -.00007	.03 -.07	3.0

TABLE 5

THE TOLERATED DOSE OF ETHYLHYDROCUPREIN AND OTHER CINCHONA DERIVATIVES BY SINGLE SUBCUTANEOUS INJECTION

Substance	Animal	Tolerated Dose		Estimated Dose per 60 Kilos (125 lb.)
		Per Gram	Per Kilo	
Ethylhydrocuprein hydrochlorid.....	Mouse.....	.00008-.0001	.08-.1	5.4
	Rabbit....	.00008-.00012	.08-.12	6.0
Quinin and urea hydrochlorid.....	Mouse.....	.00009-.00012	.09-.12	6.3
	Rabbit....	.00009-.00015	.09-.15	7.2
Hydroquinin hydrochlorid.....	Mouse.....	.00009-.0001	.09-.1	5.7
	Rabbit....	.00008-.0001	.08-.1	5.4
Quinin hydrochlorid.....	Mouse.....	.00009-.00012	.09-.12	6.3
	Rabbit....	.00009-.00018	.09-.18	8.1

Animals were infected with from 10 to 1000 times the M.L.D. of the various strains, but our best and most definite results were observed with 50 M.L.D. of a culture and most of the work was conducted with this amount.

All of the drugs were prepared in sterile distilled water, filtered, and sterilized in the Arnold sterilizer.

All the animals were kept under observation for a period of at least 6 days. Examinations were made of the majority of animals succumbing, and the blood of the heart was examined by smear and culture for pneumococci.

Intravenous Administration of Drugs.—The results of a few typical experiments in which the drugs were given intravenously 2 hours before infection are shown in Tables 6, 7, 8, and 9.

TABLE 6
PROTECTIVE VALUE OF ETHYLHYDROCUPREIN ADMINISTERED INTRAVENOUSLY 2 HOURS
BEFORE INFECTION*

BEFORE INFECTION*				
Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	12	.00004	.04	Living on 6th day
2	14	.00003	.03	
3	14	.00002	.02	
4	19	.00001	.01	Died on 5th day
5	17	.000008	.008	Died on 3rd day
6	17	.000006	.006	Died on 3rd day
7	18	.000004	.004	Died on 2nd day
8	23	.000002	.002	
9	16	Controls		
10	18			
11	18			
12	16			

* Mice infected by intraperitoneal injection with 50 M.L.D. of pneumococcus, Type I.

TABLE 7
PROTECTIVE VALUE OF HYDROQUININ HYDROCHLORID ADMINISTERED INTRAVENOUSLY
2 HOURS BEFORE INFECTION *

2 HOURS BEFORE INFECTION *				
Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	20	.00004		Living on 6th day
2	21	.00003	.04	
3	14	.00002	.03	
4	20		.02	Died on 4th day
5		.00001	.01	
6	19			
7	18	.000008	.008	Died on 3rd day
8	20	.000006	.006	
9	21	.000004	.004	
10	21	.000002	.002	
11	21			
12	19	Controls		Died on 2nd day
				Died on 3rd day
				Died on 2nd day

* Mice infected by intraperitoneal injection.

* Mice infected by intraperitoneal injection with 50 M.L.D. of pneumococcus, Type I.

TABLE 8
PROTECTIVE ACTION OF QUININ AND UREA HYDROCHLORID ADMINISTERED INTRAVENOUSLY
2 HOURS BEFORE INFECTION *

Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	19	.00004	.04	Died on 4th day
2	19	.00003	.03	Died on 3rd day
3	20	.00002	.02	Died on 3rd day
4	17	.00001	.01	Died on 2nd day
5	15	.000008	.008	
6	16	.000006	.006	
7	17	.000004	.004	
8	19	.000002	.002	
9	13	Controls		Died on 3rd day
10	16			Died on 2nd day
11	21			
12	15			

* Mice infected by intraperitoneal injection with 50 M.L.D. of pneumococcus, Type I.

TABLE 9
PROTECTIVE AND CURATIVE ACTION OF ETHYLHYDROCUPREIN HYDROCHLORID AND OTHER
CINCHONA DERIVATIVES ADMINISTERED TO RABBITS BY INTRAVENOUS INJECTION *

Test	Weight (gm.)	Substance	Dose per Kilo	Time of Injection in Relation to Inoculation	Results
1	1590	Ethylhydrocuprein.....	.01 gm.	4 hr. before.....	Living on 6th day
2	1140			2 hr. before.....	Living on 6th day
3	1275			Simultaneously.	Died on 6th day
4	1705			1 hr. after.....	Died on 4th day
5	1240			2 hr. after.....	Died on 5th day
6	1490	Quinin and urea hydrochlorid..		4 hr. before.....	Died on 3rd day
7	1350			2 hr. before.....	Died on 3rd day
8	1275			Simultaneously.	Died on 5th day
9	1320			1 hr. after.....	Died on 3rd day
10	1275			2 hr. after.....	Died on 3rd day
11	1350	Hydroquinin hydrochlorid.....		4 hr. before.....	Died on 5th day
12	1275			2 hr. before.....	Died on 4th day
13	1100			Simultaneously.	Died on 3rd day
14	1400			1 hr. after.....	Died on 3rd day
15	1250			2 hr. after.....	Died on 3rd day
16	1400	Controls.....			Died on 2nd day
17	1350				Died on 3rd day
18	1275				Died on 3rd day

* Rabbits infected by intravenous injection with 50 M.L.D. of pneumococcus, Type I.

The results of these and similar experiments in which the animals were infected with 50 M.L.D. of culture of Type I may be summarized as follows:

Of the three cinchonics used, ethylhydrocuprein hydrochlorid showed the most marked protective action against virulent pneumococci. Among mice, doses of ethylhydrocuprein hydrochlorid corre-

sponding to 0.02 gm. per kilo of body weight given 2 hours before infection protected all animals over a period of 6 days and usually much longer. The administration to rabbits of 0.01 gm. per kilo of body weight yielded similar protection.

Among mice, doses of ethylhydrocuprein hydrochlorid corresponding to 0.01-0.006 gm. per kilo of body weight prolonged life for from 1 to 3 or 4 days and did not protect indefinitely.

As a general rule, when the drug in dose of 0.02 gm. for mice and 0.01 gm. for rabbits per kilo and the pneumococci were injected within a few seconds of each other (marked "simultaneously" in the tables) the life of about 20% of the animals was prolonged indefinitely, while in the balance life was prolonged but a few days beyond that of the controls.

Both hydroquinin hydrochlorid and quinin and urea hydrochlorid showed protective value. Hydroquinin proved somewhat superior to quinin and urea hydrochlorid, and in some experiments 0.02 gm. of this salt per kilo of body weight protected mice over a period of 6 days or longer (Table 7). The percentage of protections of this degree was less than observed with ethylhydrocuprein. A dose of 0.01 gm. per kilo of body weight afforded some protection in a small percentage of mice, but smaller doses were without appreciable effect. Among rabbits the administration of hydroquinin in dose of 0.01 gm. per kilo of body weight from 2 to 4 hours previous to inoculation with pneumococci, tended to prolong life for from 1 to 4 days, but when both were given simultaneously practically no protection was afforded.

Quinin and urea hydrochlorid in large doses (0.02 to 0.04 gm. per kilo of body weight) prolonged the life of mice from 1 to 3 or 4 days in the majority of instances; smaller doses were without effect. This double salt in dose of 0.01 gm. per kilo of body weight in rabbits was practically without protective value when given from 4 hours to a few seconds before inoculation with 50 M.L.D. of pneumococcus culture.

Results with Single Intramuscular Injection.—The results of a number of experiments conducted with ethylhydrocuprein hydrochlorid, hydroquinin hydrochlorid, and quinin and urea hydrochlorid administered by single intramuscular injection are shown in Tables 10, 11, 12, and 13.

Summary.—Ethylhydrocuprein hydrochlorid in doses of from 0.02 to 0.06 gm. per kilo of body weight of mice afforded protection in the majority of experiments over a period of 6 days. In general, the

TABLE 10
PROTECTIVE VALUE OF ETHYLHYDROCUPREIN HYDROCHLORID ADMINISTERED IN SINGLE INTRA-
MUSCULAR DOSE 2 HOURS BEFORE INFECTION *

Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	19	.00006	.06	Living on 6th day
2	19	.00005	.05	Living on 6th day
3	20	.00004	.04	Died on 3rd day
4	19	.00002	.02	Died on 2nd day
5	17	.00001	.01	
6	20	.000008	.008	
7	21	.000006	.006	
8	19	.000004	.004	
9	20	Controls		
10	21			
11	18			
12	19			

* Mice infected by intraperitoneal injection with 50 M.L.D. of pneumococcus, Type I.

TABLE 11
PROTECTIVE VALUE OF HYDROQUININ HYDROCHLORID ADMINISTERED IN SINGLE INTRA-
MUSCULAR DOSE 2 HOURS BEFORE INFECTION *

Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	20	.00006	.06	Died on 3rd day
2	21	.00005	.05	
3	18	.00004	.04	
4	23	.00002	.02	
5	15	.00001	.01	
6	18	.000008	.008	
7	21	.000006	.006	Died on 2nd day
8	16	.000004	.004	
9	20	Controls		
10	21			
11	18			
12	19			

* Mice infected by intraperitoneal injection with 50 M.L.D. of pneumococcus, Type I.

TABLE 12

PROTECTIVE VALUE OF QUININ AND UREA HYDROCHLORID ADMINISTERED IN SINGLE INTRA-MUSCULAR DOSE 2 HOURS BEFORE INFECTION *

Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	15	.00006	.06	Died on 2nd day
2	17	.00004	.04	
3	15	.00003	.03	
4	21	.00002	.02	
5	17	.00001	.01	
6	20	.000008	.008	
7	15	.000006	.006	
8	18	.000004	.004	
9	16	Control		
10	19	Control		
11	21	Control		
12	18	Control		

* Mice infected by intraperitoneal injection with 50 M.L.D. of pneumococcus, Type I.

TABLE 13

PROTECTIVE AND CURATIVE ACTION OF ETHYLHYDROCUPREIN HYDROCHLORID AND OTHER CINCHONA DERIVATIVES ADMINISTERED BY INTRAMUSCULAR INJECTION IN MICE *

Test	Weight (gm.)	Substance	Dose per Kilo	Time of Injection in Relation to Inoculation	Results
1	14	Ethylhydrocuprein.....	.02 gm.	2 hr. before....	Living on 6th day
2	18			Simultaneously.	Died on 5th day
3	12			2 hr. after.....	Died on 4th day
4	17	Quinin and urea hydrochlorid..		6 hr. after.....	Died on 1st day
5	15			2 hr. before....	Died on 5th day
6	16			Simultaneously.	Died on 2nd day
7	12	Hydroquinin hydrochlorid.....		2 hr. after.....	Died on 1st day
8	22			6 hr. after.....	Died on 1st day
9	11			2 hr. before....	Died on 6th day
10	14	Quinin bisulfate.....		Simultaneously.	Died on 2nd day
11	19			2 hr. after.....	Died on 1st day
12	15			6 hr. after.....	Died on 1st day
13	24	Controls... ..		2 hr. before....	Died on 3rd day
14	18			Simultaneously.	Died on 2nd day
15	18			2 hr. after.....	Died on 1st day
16	19			6 hr. after....	Died on 1st day
17	20				
18	14				
19	16				
20	19				

* Mice infected by intraperitoneal injection with 10 M.L.D. of pneumococcus, Type I.

degree of protection was less than that observed when the drug was given intravenously. When the drug and pneumococci were given simultaneously doses of 0.04 gm., or higher, per kilo of body weight usually protected; doses of 0.02 gm., or slightly less, prolonged life for a few days among the majority of mice.

Hydroquinin hydrochlorid and quinin and urea hydrochlorid in doses of from 0.02 to 0.06 gm. per kilo of body weight tended to prolong life, and in a few instances the larger doses afforded protection over a period of at least 6 days; but both were inferior to ethylhydrocuprein hydrochlorid and the effects were much less marked than those observed when the drugs were given intravenously.

When these drugs in doses of 0.02 gm., or less, per kilo of body weight were given simultaneously with the pneumococci (50 M.L.D.), practically no protection followed.

A few experiments with quinin bisulfate showed a feeble protective value when given from 2 to 4 hours before the pneumococci.

CURATIVE VALUE

The curative action of ethylhydrocuprein hydrochlorid and other quinin derivatives was studied by injecting the drugs intravenously and intramuscularly at varying intervals after the animals had been inoculated with pneumococci or, in other words, after the infection had had a start.

Otherwise these experiments were conducted in exactly the same manner as the experiments for protective value.

Results with Intravenous Injection.—The results of a number of experiments are shown in Tables 9, 14, 15, 16, and 17.

Summary.—Our percentage of 'cures' with ethylhydrocuprein hydrochlorid when the drug was given within 2 hours after inoculation with 50 M.L.D. of culture was less than 10%. Doses ranging from 0.01 to 0.03 gm. per kilo of body weight usually prolonged life beyond that of the controls from 1 to 4 days, but ultimately the majority of the mice and rabbits succumbed.

Hydroquinin hydrochlorid in amounts as high as 0.04 gm. per kilo of body weight given from 1 to 4 hours after infection with 50 M.L.D. of culture, did not appreciably prolong life except in a few instances when the animals lived from 1 to 3 days longer than the controls.

The double salt, quinin and urea hydrochlorid, was similarly without curative value in doses as high as 0.04 gm. per kilo when given at

TABLE 14
CURATIVE ACTION OF ETHYLHYDROCUPREIN ADMINISTERED INTRAVENOUSLY 2 HOURS
AFTER INFECTION *

Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	16	.00004	.04	Living on 6th day
2	20	.00003	.03	Died on 4th day
3	14	.00002	.02	Died on 2nd day
4	15	.00001	.01	
5	13	.000008	.008	
6	14	.000006	.006	
7	12	.000004	.004	Died on 1st day
8	15	.000002	.002	Died on 2nd day
9	15	Controls		
10	16			
11	18			
12	16			

* Mice infected by intraperitoneal injection with 50 M.L.D. of pneumococcus, Type I.

TABLE 15
CURATIVE VALUE OF HYDROQUININ HYDROCHLORID ADMINISTERED INTRAVENOUSLY 2 HOURS
AFTER INFECTION *

Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	17	.00004	.04	Died on 2nd day
2	23	.00003	.03	
3	17	.00002	.02	
4	19	.00001	.01	
5	15	.000008	.008	Died on 5th day
6	22	.000006	.006	Died on 2nd day
7	24	.000004	.004	
8	20	.000002	.002	
9	21	Controls		Died on 3rd day
10	21			Died on 2nd day
11	22			Died on 3rd day
12	19			Died on 2nd day

* Mice infected by intraperitoneal injection with 50 M L. D. of pneumococcus, Type I.

TABLE 16
CURATIVE ACTION OF QUININ AND UREA HYDROCHLORID ADMINISTERED INTRAVENOUSLY 2 HOURS
AFTER INFECTION *

Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	12	.00004	.04	Died on 2nd day
2	21	.00003	.03	
3	20	.00002	.02	
4	21	.00001	.01	
5	19	.000008	.008	Died on 3rd day
6	12	.000006	.006	Died on 2nd day
7	10	.000004	.004	
8	14	.000002	.002	
9	13	Controls		Died on 3rd day
10	16			Died on 2nd day
11	21			
12	15			

* Mice infected by intraperitoneal injection with 50 M. L. D. of pneumococcus, Type II.

TABLE 17
CURATIVE ACTION OF QUININ AND UREA HYDROCHLORID ADMINISTERED INTRAVENOUSLY 2 HOURS
AFTER INFECTION *

Test	Weight (gm.)	Dose		Results
		Per Gram	Per Kilo	
1	15	.00033	0.33	Died on 6th day
2	19	.00016	0.16	Died on 6th day
3	15	.00008	0.08	Died on 5th day
4	16	.00004	0.04	Died on 5th day
5	18	.00002	0.02	Died on 4th day
6	15	.00001	0.01	Died on 4th day
7	17	.000008	0.008	Died on 3rd day
8	16	Controls	0.006	Died on 2nd day
9	21			
10	16			
11	17			
12	16			

* Mice infected by intraperitoneal injection with 10 M. L. D. of pneumococcus, Type I.

intervals of an hour or more after inoculation with 50 M.L.D. of culture. When the dose of culture was reduced to 10 M.L.D., a distinct effect was evident in the prolongation of life 4, 5, and 6 days (Table 17) among animals receiving 0.01 gm. and more per kilo of body weight.

Results with Single Intramuscular Injection.—Ethylhydrocuprein in doses ranging from 0.02 to 0.06 gm. per kilo of body weight prolonged the life of a small percentage of mice and rabbits when given within 2 hours after infection with 50 M.L.D. of culture; smaller doses in this time and similar doses given at longer periods were practically without effect (Table 13).

Hydroquinin hydrochlorid, quinin and urea hydrochlorid, and quinin bisulfate in doses ranging as high as 0.04 gm. per kilo of body weight were practically without any effect when given within 1 hour or longer after inoculation with 50 M.L.D. of culture.

Results with Multiple Intramuscular Injection.—Three of the cinchona derivatives were administered by repeated intramuscular injections with results similar to those shown in the 3 experiments detailed in Tables 18, 19, and 20.

Summary.—Ethylhydrocuprein hydrochlorid prolonged the lives of a few more mice to the 7th day or longer than did the other cinchonics. It is probable that the death of some of the mice was due to the cumulative effects of the repeated large doses.

Quinin and urea hydrochlorid ranked second in efficacy to ethylhy-

TABLE 18

RESULTS WITH REPEATED INTRAMUSCULAR INJECTIONS OF ETHYLHYDROCUPREIN HYDROCHLORID AND PNEUMOCOCCUS, TYPE I *

Test	Weight (gm.)	Dose in Each Injection		Number of Injections	Results on 7th Day †
		Per Gram	Per Kilo		
1	15	.00003	.03	4	Died on 5th day
2	22	.000025	.025	7	Living
3	23	.000016	.016	4	Died on 7th day
4	23	.000013	.013	7	Living
5	23	.00001	.01	7	Living
6	19	.000001	.001	6	Died on 7th day
7	21	.000003	.003	7	Living
8	20	.000008	.008	4	Died on 7th day

* Mice infected by intraperitoneal injection with 50 M.L.D. of culture about an hour after the first intramuscular injection of drug.

† Four controls receiving culture alone died within 48 hours.

TABLE 19

RESULTS WITH REPEATED INTRAMUSCULAR INJECTIONS OF QUININ AND UREA HYDROCHLORID AND PNEUMOCOCCUS, TYPE I *

Test	Weight (gm.)	Dose in Each Injection		Number of Injections	Results on 7th Day †
		Per Gram	Per Kilo		
1	18	.00003	.03	3	Died on 4th day
2	17	.000025	.025	5	Died on 5th day
3	18	.000016	.016	7	Living
4	24	.000013	.013	7	Living
5	19	.00001	.01	3	Died on 4th day
6	19	.000005	.006	7	Living
7	21	.000003	.003	7	Living
8	21	.000008	.008	7	Living

* Mice injected by intraperitoneal injection with 50 M. L. D. of culture about an hour after the first intramuscular injection of drug.

† Four controls receiving culture alone died within 48 hours.

TABLE 20

RESULTS WITH REPEATED INTRAMUSCULAR INJECTIONS OF HYDROQUININ HYDROCHLORID AND PNEUMOCOCCUS, TYPE I *

Test	Weight (gm.)	Dose in Each Injection		Number of Injections	Results on 7th Day †
		Per Gram	Per Kilo		
1	22	.00003	.03	7	Died on 4th day
2	24	.000015	.015	3	Died on 3rd day
3	21	.000016	.016	3	Died on 4th day
4	24	.000013	.013	4	Died on 6th day
5	10	.00001	.01	3	Died on 4th day
6	22	.000006	.006	4	Died on 5th day
7	19	.000003	.003	7	Living
8	19	.000008	.008	7	Living

* Mice infected by intraperitoneal injection with 50 M.L.D. of culture about an hour after the first intramuscular injection of drug.

† Four controls receiving culture alone died within 48 hours.

drocuprein, and indeed in the experiment shown (Table 19) carried a larger number (5) of the 8 mice beyond the 7th day.

Hydroquinin hydrochlorid demonstrated a much lower efficacy than the other two compounds. As this derivative is, according to our results, somewhat more toxic for mice and rabbits than the other com-

TABLE 21
RELATION OF TYPE OF PNEUMOCOCCUS TO THE PROTECTION AND CURATIVE ACTION OF
QUININ COMPOUNDS *

Test	Weight (gm.)	Substance	Time of Injection in Relation to Inoculation	Type I †				Type II				Type III			
				1	2	3	4	1	2	3	4	1	2	3	4
1	18	Ethylhydrocuprein hydrochlorid	2 hr. before.....				L								L
2	16		1 hr. before.....				L								D
3	14		Simultaneously.				D								D
4	16		1 hr. after.....				D								
5	22		2 hr. after.....			D		D				D			
6	18	Hydroquinin hydrochlorid	2 hr. before.....				D								
7	17		1 hr. before.....				D				L			D	
8	19		Simultaneously.					D		D				D	
9	14		1 hr. after.....			D		D		D				D	
10	18		2 hr. after.....			D		D						D	
11	16	Quinin and urea hydrochlorid	2 hr. before.....				D								
12	16		1 hr. before.....				D							D	
13	16		Simultaneously.							D				D	
14	20		1 hr. after.....			D		D						D	
15	19		2 hr. after.....			D		D						D	

D = Died. L = Living.

* Controls on Types I and II died on first day; controls on Type III died within 48 hours. All compounds were given intravenously in dose of 0.02 gm. per kilo of body weight.

† Mice were infected by intraperitoneal injection with 50 M.L.D. of the three types of pneumococci.

pounds, it is highly probable that many of the mice succumbed to the toxic effects of the drug.

RELATION OF TYPE AND VIRULENCE OF PNEUMOCOCCI TO THE PROTECTIVE AND CURATIVE ACTION OF QUININ DERIVATIVES

Cumulative experimentation with virulent cultures belonging to the serologic Types I, II, and III, show that all three are susceptible to the influence of ethylhydrocuprein hydrochlorid and other cinchona derivatives. In several of our experiments, however, Type III was found more resistant (Table 21).

In conducting these comparative tests an attempt was made to infect the animals with as nearly 50 M.L.D. of each type of pneumococcus as possible. The results of one of these comparative tests are shown in Table 18, but similar tests with other cultures belonging to the three types have not yielded exactly similar results, altho, as previously stated, Type III was generally somewhat more difficult to influence.

We have not been able thus far to carry out similar and more extensive experiments by varying the dosage of pneumococci used in infecting the animals. A few comparative experiments conducted with 10, 100, 500, and 1000 minimal lethal doses of Type I show that the

protective and curative values of ethylhydrocuprein hydrochlorid and quinin and urea hydrochlorid are considerably influenced by the severity of the infection. As previously stated, the majority of our experiments were conducted with 50 minimal lethal doses of each type, as this dose regularly killed the controls and did not appear excessive for the purpose of obtaining approximately definite results. With greater infection the protective and curative effects were much less noticeable; when 1000 minimal lethal doses were used ethylhydrocuprein was still effective to some degree, but the quinin compounds used were practically without effect.

CONCLUSIONS

The toxicity of ethylhydrocuprein hydrochlorid for mice and rabbits is slightly greater than that of other cinchona derivatives.

As a general rule, mice are found more resistant to the toxic effects of quinin derivatives than rabbits, and young rabbits more than older and heavier rabbits.

The immediately lethal dose of ethylhydrocuprein hydrochlorid in solution by intravenous injection is about 0.06 gm. and of the quinin compounds tested 0.04 to 0.06 gm. per kilo of body weight.

By intramuscular injection the lethal dose of ethylhydrocuprein hydrochlorid is about 0.6 gm. and of the quinin compounds tested about 0.5 gm. per kilo of body weight.

The tolerated dose (period of 10 days) of ethylhydrocuprein hydrochlorid by intravenous injection is about 0.04 gm. and of the quinin compounds tested about 0.05 gm. per kilo of body weight.

By intramuscular injection the tolerated dose of ethylhydrocuprein hydrochlorid is about 0.06 gm. and of the quinin compounds tested about 0.07 gm. per kilo of body weight.

By subcutaneous injection the tolerated dose of ethylhydrocuprein hydrochlorid is about 0.1 gm. and of the quinin compounds tested about 0.12 gm. per kilo of body weight.

In mice the intravenous injection of ethylhydrocuprein hydrochlorid in amount ranging from 0.02 gm. and higher per kilo of body weight affords complete protection against 50 minimal lethal doses of pneumococci given 2 hours later by intraperitoneal injection. Doses ranging from 0.01 to 0.006 gm. afford partial protection.

Among rabbits a dose of at least 0.01 gm. of ethylhydrocuprein per kilo of body weight is required to afford protection against 50 minimal lethal doses of pneumococci given 2 hours later by intravenous injection.

Hydroquinin hydrochlorid and quinin and urea hydrochlorid also possess protective power against virulent pneumococci in mice and rabbits, but to much less degree than ethylhydrocuprein hydrochlorid. Their protective power, however, increases markedly with the dose.

Ethylhydrocuprein hydrochlorid and other cinchona derivatives when intramuscularly administered afford less protection and must be given in larger doses than when administered intravenously.

When ethylhydrocuprein hydrochlorid and the quinin compounds are given at the same time as the pneumococci, protection is much less in evidence.

When ethylhydrocuprein hydrochlorid is given intravenously in amounts ranging from 0.01 to 0.3 gm. per kilo of body weight 2 hours after inoculation with 50 M.L.D. of virulent pneumococci, complete protection, or 'cure,' follows in less than 10% of animals.

Hydroquinin hydrochlorid and quinin and urea hydrochlorid in doses as high as 0.04 gm. per kilo of body weight generally fail to protect, or 'cure,' animals inoculated from 1 to 2 hours earlier with 50 M.L.D. of a culture of virulent pneumococci.

The curative effect of these compounds is less when they are administered by intramuscular injection than when they are given intravenously.

Repeated injections, especially in the case of quinin and urea hydrochlorid, are much more effective than single injections, and in some cases prolong life from 4 to 7 days or longer.

Different serologic types of virulent pneumococci possess in general similar resistance to the effects of ethylhydrocuprein and the quinin compounds. Pneumococci belonging to Type III are, however, not infrequently more resistant than Types I and II.

The results of protective and curative experiments are greatly modified according to the doses of virulent pneumococci used in inoculating the experimental animals, and further observations with smaller but regularly fatal doses of pneumococci may show a greater protective and curative value on the part of an optimal dose of the compounds.

Altho our study shows a real protective and curative value in ethylhydrocuprein in experimental pneumococcus infections, our results demonstrate a somewhat less marked antipneumococcus potency on the part of this drug than has been previously reported.* Other cinchona

* It is only fair to point out, however, that both Morgenroth and Moore obtained their best results from the use of optochin base in oil, while the present reports deal chiefly with ethylhydrocuprein hydrochlorid in water. The war has cut off our supply of the drug and we have to await a return of international sanity before we can revise our results in this particular.

derivatives are shown to possess also a certain degree of protective power in experimental infections. Further experimentation may improve the bactericidal chemotherapeutic value of ethylhydrocuprein by additions, substitutions, or other molecular modifications. In our opinion it stands today in a position somewhat similar to that of atoxyl in the treatment of experimental trypanosomiasis.

Pneumococcus infection in mice and rabbits, however, it needs scarcely be said, differs greatly from the pneumonias of man; and other factors, especially the toxemic complex and the mechanism of crisis (the development of critical immunity and the danger of critical death), have to be considered in the therapeutics of the latter malady. Nevertheless, stress may be laid on the prolongation of life (Table 19) in infected mice receiving repeated injections of nontoxic doses of quinin and urea hydrochlorid—and to a slightly greater extent (Table 18) in those treated with ethylhydrocuprein. For such prolongation in man gives time¹⁷ for the development of the natural immunity which is manifested by critical recovery in untreated cases and by gradual defervescence (lysis) in cases treated with quinin.

It must also be remembered in this connection in so far as the facts here reported may be considered to bear on the value of quinin compounds in the treatment of pneumonias in man—that the use of these agents as advocated by one of us forms but one element in a therapeutic plan,¹⁸ and that, at least until some more potent pneumococcide in vitro than even optochin is found, it is the plan as a whole, and not any single feature only, that is offered for the consideration of practicing physicians.

¹⁷ Cohen, *New York Med. Jour.*, 1916, 103, p. 1065.

¹⁸ *Ibid.*, p. 1057.

THE INFLUENCE OF QUININ AND UREA HYDRO-
CHLORID, ETHYLHYDROCUPREIN, AND OTHER
CINCHONA DERIVATIVES ON LEUKOCY-
TOSIS AND ON PHAGOCYTOSIS OF
PNEUMOCOCCI IN VITRO*

STUDIES IN PNEUMONIA, IV.

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In the research concerning the action of cinchona derivatives in pneumonias, of which an additional phase is reported in the present paper, the question of the effect of these drugs on leukocytic, and especially on phagocytic action, was one of the problems early formulated. The clinician (S. S. C.) had long ago observed and recorded the fact that contrary to the general impression—and indeed to his own expectation—the administration of quinin, in any form and by any method, to a patient in the early stages of a lobar or lobular pneumonia, not only did not reduce the numbers or the apparent activity of the leukocytes, but—so far as one might judge in an infection normally accompanied with a progressive increase in the white-cell count—actually seemed to increase them. This apparent increase in leukocytosis was especially marked in cases treated by intramuscular injections of quinin and urea hydrochlorid, and several case reports illustrative of this fact have been published in the various clinical papers of the observer named.¹ The clinical observations on the differential count of leukocytes did not, however, show much change; or, at least, the changes were not sufficiently regular in the limited number of observations made, and under the great variety of clinical conditions, to warrant definite statement. On the whole, the increase seemed to affect chiefly the polymorphic and, to a less degree, the large mononuclear cells; but no stress could be, or had been, laid on this particular phase of the result.

In the later stages of lobar and lobular pneumonia, while there has

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¹ Jour. Infect. Dis., 1917, 20, p. 40. Am. Jour. Med. Sc., 1912, 143, pp. 42, 43. Jour. Am. Med. Assn., 1913, 61, p. 107 (Charts I and II). Internat. Clin., Series 22, 3, p. 62 (Chart II).

been no diminution of leukocytes that could be observed clinically, at least none that could be conclusively demonstrated, neither has there been any positively marked increase. Further studies will be directed toward these points, especially with regard to serologic types of microorganisms and in the light of the experimental facts reported in this paper.

The clinical observations referred to have, however, offered a sufficiently promising basis to warrant experimental study, especially when taken in connection with the general importance of phagocytosis in immunity in acute infections, particularly in those dependent on the pneumococcus. Other observers have preceded us in some portions of this field.

Tschistovitsch,² in 1904, found that in experimental pneumonia of the dog and in human pneumonia, phagocytosis was marked and he ascribed the crisis to this process. Kruse and Pansini³ likewise described the phagocytosis of pneumococci in vivo; Lamar and Meltzer⁴ found active phagocytosis of virulent pneumococci in the pulmonary exudates of experimentally produced pneumonias in dogs on the 2nd day following intrabronchial insufflation. On the 4th day practically all the cocci were found within cells. Similar results were reported by Winternitz and Hirschfelder⁵ in the experimentally produced pneumonias of rabbits; these investigators also showed that depression of the leukocytic mechanism by injections of benzol greatly increased the mortality,⁵ while injection of toluol, which tends to increase the number of leukocytes and cause hyperplastic changes in the bone marrow, was shown by Kline and Winternitz⁶ to increase resistance in experimentally produced pneumococcus pneumonia. Winternitz and Kline⁷ also demonstrated by further experiments, the rôle of the leukocyte in the immunity reaction in pneumonia, and by intravital staining methods with trypan blue showed that up to the 7th day the majority of leukocytes in experimental lesions produced by virulent pneumococci were in a living state. Bull⁸ also described the phagocytosis of virulent pneumococci in experimental pneumonia following their previous agglutination, and regards agglutination and subsequent phagocytosis as important factors in immunity in pneumococcus infections.

On the other hand, opsonic studies in vitro by Rosenow,⁹ Tschistovitsch and Yurevitsch,¹⁰ and Wadsworth¹¹ tended to show that virulent pneumococci are insusceptible to phagocytosis. Wadsworth¹¹ in a study of 25 pneumonic lungs found very few bacteria in the exudate and very little evidence of phagocytosis.

² An. de l'Inst. Pasteur, 1890, 4, p. 285; 1904, 18, p. 304.

³ Ztschr. f. Hyg. u. Infektionskr., 1891-1892, 11, p. 279.

⁴ Jour. Exper. Med., 1912, 15, p. 133.

⁵ Ibid., 1913, 17, p. 657.

⁶ Ibid., 18, p. 50.

⁷ Ibid., 1915, 21, pp. 311, 320.

⁸ Ibid., 1915, 22, p. 457; 1916, 24, p. 7.

⁹ Jour. Infect. Dis., 1907, 4, p. 285.

¹⁰ Ann. de l'Inst. Pasteur, 1908, 22, p. 611.

¹¹ Jour. Exper. Med., 1912, 16, p. 54.

Newfeld and Haendel,¹² Rosenow,¹³ Potter and Krumweide,¹⁴ and Hektoen,¹⁵ and others found that the opsonic activity of the serum and the phagocytic activity of the leukocytes were increased in pneumonia and other infectious diseases, but Seligmann and Klopstock,¹⁶ Boettcher,¹⁷ and Strouse¹⁸ were unable to substantiate the claim that this change in the leukocytes themselves is an important factor. Strouse¹⁹ through animal experiments concluded that altho opsonic immunity is produced in pneumonia, it is not the only means of defense possessed by the body, and by itself cannot explain the crisis.

The object of the present investigation was to determine the possible influence of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchona derivatives on leukocytosis and on phagocytosis of virulent pneumococci in vitro, as possibly shedding additional light on the action of these compounds in the treatment of the acute pneumonias. A report of further studies bearing on the influence of these derivatives on the phagocytosis of pneumococci in vivo will be given later. Manwaring,²⁰ Wilson,²¹ and Grünspan²² showed that strong dilutions of the quinin salts tend to inhibit phagocytosis of micro-organisms in general, while weaker solutions act as stimulants. Wright²³ reported that ethylhydrocuprein administered to man does not increase the opsonic powers of the blood serum for pneumococci. Our experiments, on the other hand, show that weak dilutions of this derivative and other quinin compounds, materially facilitate the phagocytosis of virulent pneumococci.

METHOD OF STUDY

Washed rabbit leukocytes were used exclusively and all the work reported here was done with those obtained by intrapleural injection of sterile aleuronat. Leukocytes obtained in this manner proved more actively phagocytic than those in the peripheral blood, as pointed out by Tunnicliff some years ago.²⁴ After one washing with warm sterile salt solution the cells were resuspended in warm salt solution with an attempt to make the emulsions of successive experiments approximately equal in their content of polymorphonuclear neutrophiles.

Pure cultures of pneumococci belonging to Types I, II, and III were employed; most of the work was conducted with Type I.

¹² Ztschr. f. Immunitätsf., 1909, 3, p. 159.

¹³ Jour. Infect. Dis., 1906, 3, p. 683.

¹⁴ Ibid., 1907, 4, p. 601.

¹⁵ Jour. Am. Med. Assn., 1911, 57, p. 1579.

¹⁶ Ztschr. f. Immunitätsf., 1909, 4, p. 103.

¹⁷ Deutsch. Arch. f. klin. Med., 1909, 98, p. 93.

¹⁸ Jour. Exper. Med., 1909, 11, p. 743.

¹⁹ Ibid., 1911, 14, p. 109.

²⁰ Ibid., 1907, 9, p. 473.

²¹ Am. Jour. Physiol., 1907, 19, p. 445.

²² Centralbl. f. Bakteriöl., I, O., 1908, 48, p. 444.

²³ On Pharmaco-therapy and Preventive Inoculation Applied to Pneumonia in the African Native, 1915, 16.

²⁴ Tr. Chicago Path. Soc., 1911, 8, p. 208.

The cocci were grown in blood dextrose broth for from 18 to 24 hours; carefully pipetted into centrifuge tubes; washed once with warm sterile salt solution and resuspended in salt solution until an emulsion was secured containing approximately a billion or more cocci to the cubic centimeter. As 24-hour cultures are always too sparse, this enriching by centrifugation was necessary.

The tests were conducted by mixing in small test tubes 0.1 c.c. of a dilution of quinin derivative or the serum of a rabbit after the administration of a compound, and 0.1 c.c. of the pneumococcus emulsion, and incubating the mixture at 37 C. for 1 hour. At the end of this time 0.1 c.c. of leukocytic emulsion was added to each tube, gently mixed, the whole reincubated for 1 hour, and then duplicate smears prepared and stained. During the second period of incubation the tubes were gently shaken several times to maintain a mixture of cells and cocci.

Two observers counted 100 cells on each slide, noting the percentage of phagocytes; no attempt was made to count the number of cocci within the cells.

Numerous controls with cocci, salt solution, and leukocytes and with normal sera, cocci, and leukocytes, were included.

All dilutions were made with 0.85% sodium chlorid in distilled water.

SPONTANEOUS PHAGOCYTOSIS OF PNEUMOCOCCI

Under the conditions detailed, fairly well-marked spontaneous phagocytosis was noted with different cultures of various types of pneumococci, all of which possessed some virulence for mice and rabbits, as shown in Table 1.

All these cultures were considerably more virulent for white mice according to weight, by intraperitoneal injection.

TABLE 1
SPONTANEOUS PHAGOCYTOSIS OF TYPES OF PNEUMOCOCCI AND ITS RELATION TO VIRULENCE OF PNEUMOCOCCI FOR RABBITS (INTRAVENOUS INJECTION)

Types of Pneumococci	Virulence (72 hours) *	Phagocytic Indices
Type I (Culture A).....	0.10 c.c.	3-5.5
Type I (Culture B).....	0.02 c.c.	2-3.5
Type II (Culture A).....	0.05 c.c.	2-4.0
Type II (Culture B).....	0.02 c.c.	3-4.0
Type III (Culture A).....	0.12 c.c.	3-5.5

* 24-hour dextrose-blood-broth cultures.

INFLUENCE OF CINCHONA DERIVATIVES ON PHAGOCYTOSIS OF PNEUMOCOCCI OF TYPES I, II, AND III

The results observed with 1:1000, 1:10,000, and 1:100,000 dilutions of quinin and urea hydrochlorid, ethylhydrocuprein hydrochlorid,

and other cinchona derivatives, and virulent pneumococci belonging to the first three serologic types, are shown in Tables 2, 3, and 4.

Summary.—Allowance being made for spontaneous phagocytosis, our results indicate that cinchona derivatives facilitate the phagocytosis of pneumococci in vitro.

TABLE 2

BACTERIOTROPIC VALUE OF QUININ AND UREA HYDROCHLORID, ETHYLHYDROCUPREIN, AND OTHER CINCHONA DERIVATIVES FOR PNEUMOCOCCUS TYPE I *

Quinin Derivatives	Phagocytic Indices †		
	1:1000	1:10,000	1:100,000
Ethylhydrocuprein hydrochlorid.....	7.5	8.5	9.0
Quinin and urea hydrochlorid.....	7.0	9.0	9.0
Hydroquinin hydrochlorid.....	3.5	8.0	7.0
Quinin bisulfate.....	2.0	5.0	8.0
Quinin hydrochlorid.....	3.0	4.0	10.0
Quinin salicylate.....	3.0	5.5	8.0
Quinin (alkaloid).....	2.0	3.0	6.0

* Virulence for rabbits in 72 hours by intravenous injection, about 0.1 c.c.

† Controls showed a spontaneous phagocytosis of 3-5.5%.

TABLE 3

BACTERIOTROPIC VALUE OF QUININ AND UREA HYDROCHLORID, ETHYLHYDROCUPREIN, AND OTHER CINCHONA DERIVATIVES FOR PNEUMOCOCCUS TYPE II *

Quinin Derivatives	Phagocytic Indices †		
	1:1000	1:10,000	1:100,000
Ethylhydrocuprein hydrochlorid.....	6.5	8.0	10.5
Quinin and urea hydrochlorid.....	8.0	9.5	11.0
Hydroquinin hydrochlorid.....	7.0	9.0	11.5
Quinin bisulfate.....	3.5	6.0	9.0
Quinin hydrochlorid.....	5.0	5.0	8.5
Quinin salicylate.....	6.0	7.0	9.5
Quinin (alkaloid).....	3.0	3.5	4.5

* Virulence for rabbits in 72 hours by intravenous injection, about 0.03 c.c.

† Controls showed a spontaneous phagocytosis of about 3%.

Ethylhydrocuprein hydrochlorid facilitated the phagocytosis of all three types of pneumococci, especially Type III, but with Types I and II it was excelled by quinin and urea hydrochlorid.

Hydroquinin hydrochlorid and several quinin salts also facilitated

TABLE 4
BACTERIOTROPIC VALUE OF QUININ AND UREA HYDROCHLORID, ETHYLHYDROCUPREIN, AND OTHER
CINCHONA DERIVATIVES FOR PNEUMOCOCCUS TYPE III *

Quinin Derivatives	Phagocytic Indices †		
	1:1000	1:10,000	1:100,000
Ethylhydrocuprein hydrochlorid.....	7.0	10.0	12.5
Quinin and urea hydrochlorid.....	6.5	8.0	9.5
Hydroquinin hydrochlorid.....	5.0	5.0	6.5
Quinin bisulfate.....	4.5	6.0	7.5
Quinin hydrochlorid.....	7.0	9.0	11.5
Quinin salleylate.....	5.0	7.5	9.5
Quinin (alkaloid).....	4.0	4.5	5.5

* Virulence for rabbits in 72 hours by intravenous injection, about 0.12 c.e.

† Controls showed a spontaneous phagocytosis of about 4.5%.

phagocytosis to a greater or less degree. The degree of phagocytosis observed with the alkaloid alone was scarcely greater than the degree of spontaneous phagocytosis.

In all instances the highest dilution (1:100,000) gave the best results. A dilution of 1:100, particularly of the alkaloid quinin, produced marked degenerative changes in the leukocytes with practically no phagocytosis.

BACTERIOTROPIC ACTION OF THE SERA OF RABBITS RECEIVING INTRA-MUSCULAR INJECTIONS OF QUININ COMPOUNDS AND OTHER CINCHONA DERIVATIVES

The bacteriotropic value of the sera of a number of normal rabbits was tested; then small doses of quinin and urea hydrochlorid, ethylhydrocuprein hydrochlorid and other cinchona derivatives were intramuscularly injected and similar tests of the sera made 2, 4, and 24 hours later.

The majority of these tests were conducted with a culture of pneumococcus belonging to Type I, a few experiments having shown that with Types II and III similar results were to be observed.

The results of a number of experiments are shown in Tables 5 and 6. The quantities of the various cinchona derivatives injected are shown in the tables; also the degree of phagocytosis found with the normal sera before the injections had been made and at intervals of 2, 4, and 24 hours later. All of these sera were placed in a refrigerator over night and used the following day without inactivation by heat.

TABLE 5

BACTERIOTROPIC VALUE OF RABBIT SERA FOR PNEUMOCOCCUS TYPE I AFTER ADMINISTRATION OF VARIOUS CINCHONA DERIVATIVES

Test	Weight (gm.)	Amount per Kilo (gm.)	Before Injection		2 hr. After Injection		4 hr. After Injection		24 hr. After Injection	
			1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100
After Administration of Ethylhydrocuprein Hydrochlorid										
1	1355	.002	2.3	11.0	6.4	10.0	8.0	17.0	8.5	30.0
2	1700	.001	2.2	8.2	6.4	11.0	3.5	14.5	5.5	20.0
After Administration of Quinin and Urea Hydrochlorid										
1	1255	.002	3.2	5.0	17.5	23.5	10.5	11.5	8.0	14.0
2	1200	.001	2.1	6.2	14.0	17.0	14.0	16.0	10.0	9.0
After Administration of Hydroquinin Hydrochlorid										
1	1420	.002	4.0	6.1	4.0	10.0	11.0	13.0	9.0	14.0
2	1350	.001	4.0	9.0	5.0	8.0	4.0	6.0	3.0	3.5
After Administration of Quinin Hydrochlorid										
1	1400	.002	2.0	8.0	4.0	10.0	8.0	13.0	7.0	11.0
2	1560	.001	2.5	6.5	4.5	11.5	12.0	15.0	9.5	12.0

TABLE 6

BACTERIOTROPIC VALUE OF RABBIT SERA FOR PNEUMOCOCCI AFTER ADMINISTRATION OF ETHYLHYDROCUPREIN HYDROCHLORID

Test	Weight (gm.)	Amount per Kilo (gm.)	Before Injection		2 hr. After Injection		4 hr. After Injection		24 hr. After Injection	
			1:10	1:100	1:10	1:100	1:10	1:100	1:10	1:100
For Type II										
1	1600	.002	2.0	6.0	8.0	15.0	9.0	12.0	7.0	13.5
2	1250	.001	2.0	8.0	6.5	12.0	7.5	11.5	6.5	12.0
For Type III										
1	1700	.002	1.5	4.0	4.5	10.5	5.0	12.0	6.0	11.5
2	1350	.001	2.0	4.5	5.0	11.0	4.5	10.5	4.0	10.0

The quantities of cinchona derivatives injected were relatively small and corresponded to doses of 0.12 and 0.06 gm., respectively, per 60 kilos (about 125 pounds) of body weight.

Summary.—As shown in the tables, normal rabbit serum appeared to facilitate in some instances the phagocytosis of pneumococci. A spontaneous phagocytosis ranging from 3 to 5.5% was expected with these cultures; the 1:100 dilutions of sera almost invariably yielded higher percentages of phagocytosis than the 1:10 dilutions. The sera of normal rabbits varied in pneumococcotropic value under identical experimental conditions as shown in the different tables.

Two hours after the intramuscular injection of quinin and urea hydrochlorid, ethylhydrocuprein hydrochlorid, and other cinchona derivatives, the bacteriotropic value of the sera for pneumococci was appreciably increased. At the end of 24 hours the values were still higher than before injection, but usually subsiding. In practically all instances the dilutions of serum of 1:100 gave higher values than those of 1:10.

Quinin and urea hydrochlorid and ethylhydrocuprein hydrochlorid yielded particularly definite results, each surpassing the other at certain points. The results with hydroquinin hydrochlorid were much less marked.

As a general rule, the sera of those rabbits receiving the larger of the two doses of each compound, yielded the higher bacteriotropic values.

MECHANISM OF THE ACTION OF CINCHONA DERIVATIVES

When large numbers of pneumococci were exposed to dilutions of ethylhydrocuprein hydrochlorid, quinin and urea hydrochlorid, and hydroquinin hydrochlorid of 1:1000 for 1 hour at 37 C., then washed 3 times with warm normal salt solution by centrifugation, and exposed to the leukocytes for an hour at 37 C., higher phagocytic indices were found than when the cocci were mixed with leukocytes without previous exposure to solutions of the cinchonics. These results may be ascribed to a lowering of the resistance of the cocci by the drugs, which in the case of ethylhydrocuprein, may have amounted to an actual killing of large numbers, as shown in bactericidal tests by the centrifuge method.²⁶

Experiments conducted in the routine manner, by exposure of the cocci to dilutions of the cinchonics of 1:1000, 1:10,000, and 1:100,000

²⁶ Cohen, Kolmer, and Heist, *Jour. Infect. Dis.*, 1917, 20, p. 40.

followed by the addition of leukocytes may be contrasted with similar experiments in which the cocci had been washed with warm normal salt solution before mixture with the leukocytes. Higher phagocytic indices were shown in the former series of tests, which would seem to indicate that the high dilutions of the quinin compounds stimulated the leukocytes in their phagocytic activity.

As previously mentioned, low dilutions of these compounds acted in an opposite manner, retarding phagocytosis through degenerative changes induced in the leukocytes.

INFLUENCE OF QUININ AND UREA HYDROCHLORID, ETHYLHYDROCUPREIN HYDROCHLORID, AND OTHER CINCHONICS ON THE TOTAL LEUKOCYTES

Intramuscular injections of cinchona derivatives dissolved in normal salt solution increased the total number of leukocytes in the peripheral blood of white rats.

After preliminary leukocyte counts had been made on 2 successive days of several series of rats, a number of quinin compounds were administered intramuscularly and intravenously in dosage according to body weight. Subsequent counts were made 2, 4, 24, 48, 72, and 96 hours later.

In all instances the rats were kept at ordinary room temperature and counts made after a period of fasting, in order to reduce to a minimum the influence of other factors.

Doses ranging from 0.03 to 0.001 gm. per kilo. of body weight in 0.5 c.c. of normal salt solution were injected into the muscles of the thigh.

Several controls, consisting of the intramuscular injection of normal salt solution to determine the influence of trauma alone, were included.

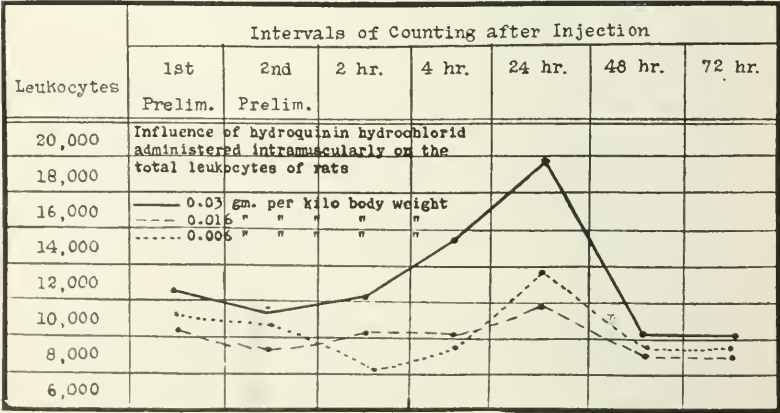
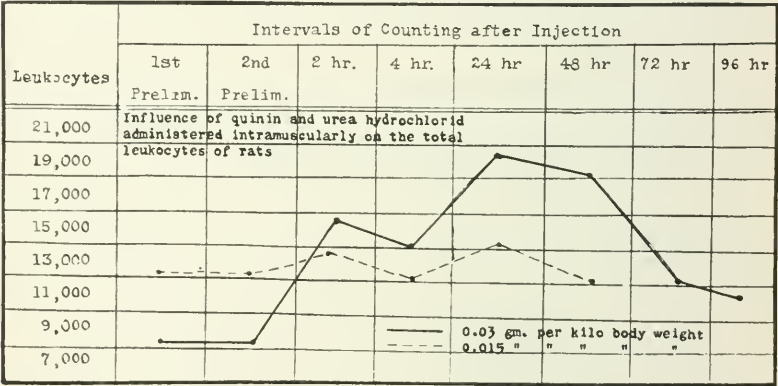
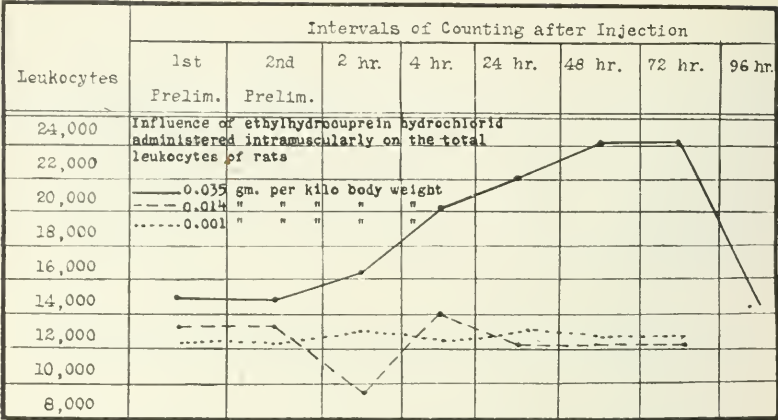
The influence of the following cinchonics was studied in this manner: ethylhydrocuprein hydrochlorid, and quinin and urea hydrochlorid, hydroquinin hydrochlorid, quinin hydrobromid, quinin lactate, quinin tannate, and quinin salicylate.

The results observed with the first three compounds are shown in the accompanying charts:

Summary.—As shown in the charts, the total leukocytes of normal rats varied from 8000 to 12,000 per cubic millimeter of blood, the number being usually nearer the latter figure. For this reason preliminary counts were necessary on each individual rat, and the effects were noted by comparing counts made after injection, with the preliminary counts.

The control rats generally showed a slight leukocytosis (increase of 1000 to 2000 cells), reaching the maximum at the end of 24 hours, followed by a rapid drop to normal.

As a general rule, the number of leukocytes was found to have



increased 4 hours after injection with these compounds, with further increases up to 48 and 72 hours; after this time the number of leukocytes gradually dropped to normal.

The degree of leukocytosis generally bore a relation to the dose injected, the greatest rise in the number of leukocytes following the administration of the largest dose.

Ethylhydrocuprein hydrochlorid induced the highest degree of leukocytosis; but well-marked leukocytosis also followed the injection of quinin and urea hydrochlorid and hydroquinin hydrochlorid. Quinin lactate and quinin salicylate produced lesser degrees of leukocytosis.

The intravenous injection of these compounds produced lesser degrees of leukocytosis; local irritation due to the deposit of the compounds in the tissues was probably a cause for the higher leukocytosis noted with the intramuscular injections.

CONCLUSIONS

Pneumococci possessing some degree of virulence for rabbits and mice may undergo spontaneous phagocytosis by the exudative leukocytes of rabbits.

Ethylhydrocuprein hydrochlorid, quinin and urea hydrochlorid, and other compounds or derivatives of quinin in high dilutions, accelerate the phagocytosis of pneumococci by rabbit leukocytes.

Low dilutions of cinchona derivatives retard phagocytosis and induce degenerative changes in leukocytes.

The administration of ethylhydrocuprein hydrochlorid, quinin and urea hydrochlorid, and other quinin derivatives to rabbits by intramuscular injection increases the phagocytic powers of the sera for pneumococci.

It is probable that these quinin derivatives in high dilution accelerate phagocytosis by reducing the virulence and resistance of the pneumococci and also by stimulating the phagocytic activities of the leukocytes.

The intramuscular injection of ethylhydrocuprein hydrochlorid, quinin and urea hydrochlorid, and other cinchona derivatives in rats increases the total number of leukocytes in the peripheral blood. Lesser degrees of leukocytosis follow the intravenous injection of these compounds.

These experiments indicate that probably a part of the curative effect of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchonics in pneumococcus infections is to be ascribed to their influence on phagocytosis.

ALLERGIC SKIN REACTIONS IN PNEUMONIA TO TYPE STRAINS OF PNEUMOCOCCI *

STUDIES IN PNEUMONIA, V

EDWARD STEINFELD AND JOHN A. KOLMER

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Allergic skin reactions in pneumonia have recently been studied during the course of immunologic investigations in this disease. Clough,¹ in 1915, studied allergy in pneumonia with the dried and ground residue of 18-hour extracts of washed pneumococci before and after precipitation with absolute alcohol. Ophthalmic, cutaneous, and intracutaneous tests with these extracts on persons with pneumonia and controls yielded varying and inconstant results attributable to the irritant qualities of the extracts, so that Clough was of the opinion that it is not possible to demonstrate a condition of hypersensitiveness to pneumococcus protein in patients with pneumonia by these tests.

In the autumn of 1915, we began the study of allergic reactions in pneumonia with suspensions of washed and heat-killed pneumococci, our object being to determine whether such emulsions were suitable for these tests and whether a condition of skin hypersensitiveness to pneumococcus protein develops in pneumonia, and if so, to apply anaphylactogens prepared of different types of pneumococci in a study of the specificity of allergic reactions in this disease as controlled by a bacteriologic diagnosis of the type or types of pneumococci found in the sputum. During the course of this investigation Weil and Torrey² reported the results of their work using Dale's method and an extract of pneumococci prepared by autolyzing the cocci in distilled water for 2 hours at 37 C. and heating at 60 C. for 1 hour. Weil and Torrey found that the blood of persons suffering with pneumonia contains a sensitizing antibody during the course of the disease, but none after the crisis. These anaphylactic reactions were found more general for

* Received for publication November 27, 1916.

¹ Bull. Johns Hopkins Hosp., 1915, 26, p. 37.

² Jour. Exper. Med., 1916, 23, p. 1.

the whole pneumococcus group than has been found to be the case with agglutination and protection tests. Weil³ injected from 0.1 to 0.2 c.c. of this extract into the skins of persons suffering with pneumonia and found no reactions during the course of the disease while "after subsidence a considerable percentage presented a reaction."

METHOD OF STUDY

Intradermal skin tests with anaphylactogens corresponding to Types I, II, and III were conducted in a group of persons suffering with lobar pneumonia both before and after the crisis, in whom a bacteriologic diagnosis of the type or types of infecting pneumococci present in the sputum had been made by the agglutination test; also in a number of healthy persons and those suffering with various chronic ailments not referable to the respiratory system.

Preparation of the Anaphylactogens.—Pure cultures of pneumococci highly virulent for mice and rabbits and belonging to serologic types I, II, and III, were grown in flasks of specially prepared dextrose broth for 48 hours; the cocci of each type were then removed and washed twice with sterile normal salt solution by centrifugation in order to remove toxic substances present in the medium. The three lots of washed cocci were then suspended in sterile salt solution, shaken mechanically, and the emulsion diluted with sufficient salt solution to make about 2 billion cocci to the cubic centimeter. Each emulsion was then heated in a water bath at 60 C. for 1 hour, cultured for sterility, and preserved with 0.2% tricresol.

Tests and Reactions.—In conducting the tests 0.1 c.c. of each emulsion was injected intracutaneously. The three injections were made at the one time in each patient.

All reactions were read 48 hours after injection. During the first 24 hours the majority of persons, both controls and those suffering with pneumonia, showed a narrow zone of hyperemia about each injection which had largely subsided in 48 hours, except in those whose reactions were interpreted as positive. Areas of erythema less than 1 c.c. in diameter and even accompanied by a small papule which regularly disappeared in 72 hours, were regarded as possibly due to irritation and negative reactions.

Positive reactions were those marked by the formation of a definite papule with an area of erythema greater than 1 c.c. in diameter and accompanied by slight edema. These reactions persisted for from 4 to 5 days and gradually disappeared. Pustular reactions did not occur in any instance.

There were no subjective symptoms beyond slight burning pain within a few hours after injection.

RESULTS WITH HEALTHY PERSONS AND THOSE SUFFERING WITH DISEASES OTHER THAN PNEUMONIA

A large number of tests with the three anaphylactogens were made on healthy persons and those suffering with various chronic ailments not involving the chest. In all instances the skin reactions were regarded as negative and uninfluenced by the presence of various types

³ Ibid., p. 11.

of pneumococci in the upper air passages. As previously stated, many of these persons presented mild reactions of erythema which disappeared in 48 hours, but these were no greater in our experience than those observed to follow other cutaneous reactions as in the luetin test. For this reason we believe that the anaphylactogens as prepared were free to a large extent of toxic or irritant substances.

RESULTS IN PERSONS WITH PNEUMONIA

The results observed in a group of 20 cases are shown in the accompanying table.

TABLE 1
ALLERGIC SKIN REACTIONS IN PNEUMONIA PATIENTS

No.	Age (yr.)	Clinical Diagnosis	Type of Pneumonia	Day of Disease	Relative to Crisis	Allergic Reactions		
						Type I	Type II	Type III
1	45	Bronchopneumonia* Double lobar	None present	10	Lysis	—†	—	—
2	34		II	5	Before	—	—	—
3	40		II	27	Lysis	—	—	—
4	23		I	10	Lysis	—	—	—
5	19		I	13	2d day after	+	—	+
6	33	Lobar pneumonia†	Not examined	26	Lysis	—	—	—
7	34		I and II	8	Before	—	—	—
8	34		IV	10	3d day after	—	—	+
9	40		IV	7	Same day	—	—	—
10	35		I and III	Unknown	9th day after	—	—	+
11	58		II	3	Before	—	—	—
12	35		II	13	Before	—	—	—
13	36		I and II	39	Lysis	—	+	+
14	51		I and II	3	Before	—	—	—
15	35		I	32	23d day after	—	—	—
16	23		I and II	24	16th day after	—	—	—
17	28		Not examined	14	5th day after	—	—	—
18	31		I and II	12	5th day after	—	+	—
19	23		I and IV	9	4th day after	+	—	—
20	32		IV	9	Before	—	—	—

* M. catarrhalis.

† No. 3 developed empyema. Nos. 6 and 13 doubtful.

‡ — = negative skin reaction. + = positive skin reaction.

As shown in the table, of 17 cases in which a diagnosis of the pneumococci present in the sputum had been made, the following types were found: Type I (alone) in 3 cases; Type II (alone) in 4 cases; Type IV (alone) in 3 cases; Types I and II in 5 cases; Types I and III in 1 case; and Types I and IV in 1 case.

Of 19 cases regarded clinically as lobar pneumonia, 6 or 30% gave positive reactions with one or more of the anaphylactogens.

In all instances the positive reactions occurred after the crisis (in one case, No. 13, there was no crisis and the positive reactions occurred on the 39th day of the disease). In our series the earliest reaction was observed on the 10th day of the disease and the 3rd after the crisis.

The positive skin reactions did not correspond strictly to the types of pneumococci found in the sputum, altho we may have overlooked other types in the sputum. Case 5 showed Type I in the sputum and reacted in the skin to the protein of Types I and III; Case 8 showed Type IV in the sputum and reacted to Type III; Case 10 showed Types I and III in the sputum and reacted to Type III; Case 13 showed Types I and II in the sputum and reacted to Types II and III; Case 18 showed Types I and II in the sputum and reacted to Type II; Case 19 showed Type I and IV and reacted to Type I (Type IV not included in the skin tests).

Further studies must determine how long this condition of hypersensitiveness persists and its relation to immunity in pneumonia.

As the reactions are not observed until after the crisis and as they apparently do not occur according to the types of pneumococci found in the sputum, these tests possess no appreciable practical value.

SUMMARY

Allergic reactions were observed in 30% of a series of cases of lobar pneumonia following the intradermal injection of washed and heat-killed pneumococci.

True reactions were not observed among normal persons or those suffering with various chronic diseases, and the presence of pneumococci in the upper air passages during health apparently does not sensitize in so far as this condition is detectable by skin tests.

All reactions occurred after the crisis or after the infection had been present over a prolonged period.

There was no constant relation between reactions to the protein of various types of pneumococci and the types found in the sputum. It is probable that the allergic reactions to pneumococcus protein are of a more general character than the agglutination reactions.

THE NONFILTRABILITY OF TYPHUS-FEVER VIRUS *

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In a communication dealing with the etiology of typhus fever, Plotz¹ presented a review of the evidence which led him to believe that the virus is nonfiltrable. No effort was made at that time to give all the facts leading to this conclusion. It is the purpose of my paper to analyze the previous work, and to present new evidence bearing on the nonfiltrability of typhus virus.

The basis for the assertion that the virus is filtrable is a single, very doubtful, experiment of Nicolle, Conon, and Conseil.² As additional support, the single incomplete experiment of Wilder³ is usually quoted. Thus, Wolbach⁴ classified typhus among the filtrable viruses on such evidence; so did Löffler⁵ and Lipschütz.⁶ R. Kraus⁷ stated dogmatically that typhus-fever virus was filtrable, while Park and Williams⁸ asserted that there was "the possibility of the virus having a filtrable stage." Jordan⁹ quite recently stated that "inoculation with filtered blood does, however, render monkeys refractory to further infection, according to the testimony of several observers."

In reviewing the original sources of the assumption that the virus is filtrable I shall analyze Nicolle's work first. All his work on this problem may be summarized in a résumé of 5 series of experiments.

The first series comprised 4 monkeys — 1 injected with unfiltered, the other 3 with filtered typhus serum. All failed to react, except 'Bonnet 47,' which was injected with filtered serum; this animal showed an elevation of one-half degree (Centigrade) between the 16th and 18th days after inoculation. After a time all these monkeys were reinoculated with typhus virus and all except Bonnet 47 developed the

* Received for publication December 2, 1916. Work done under the tenure of a George Blumenthal Jr. fellowship in pathology.

¹ Plotz, Olitsky, and Baehr, *Jour. Infect. Dis.*, 1915, 17, p. 1.

² *Ann. de l'Inst. Pasteur*, 1911, 25, p. 97. *Compt. rend. Acad. d. sc.*, 1911, 153, p. 1522.

³ *Jour. Infect. Dis.*, 1911, 9, p. 9.

⁴ *Jour. Med. Research*, 1912-1913, 22, p. 1.

⁵ *Tr. Internat. Cong. Med.*, London, 1913, Lect. 4, p. 35.

⁶ Kolle u. Wassermann, *Handb. d. pathogen. Mikroorganismen*, 1913, 8, p. 353.

⁷ *Wien. klin. Wchnschr.*, 1914, 27, p. 925.

⁸ *Pathogenic Microorganisms*, 1914.

⁹ *Text-Book of General Bacteriology*, 1916.

fever. From this single experiment (which he has never been able to confirm), Nicolle concluded that the virus is filtrable.

In the light of my experience it appears that Nicolle was dealing with avirulent blood — certainly his control test shows this. Also, the first reaction in Bonnet 47 can hardly be called typhus fever — a rise in temperature of 1 to 2 degrees for 1 or 2 days is quite common in normal monkeys. The animal remained refractory to a subsequent injection of virulent blood either because it was a naturally immune monkey, or because it was not inoculated often enough. Anderson and Goldberger¹⁰ showed that 22½% of the monkeys used failed to react to virulent blood; 31½% resisted 3 or more successive inoculations, and were not immunized by these repeated injections. This conforms with our experience at Mount Sinai Hospital Laboratory. A conclusion based on Nicolle's uncertain and uncontrolled series of experiments seems to me quite unwarranted.

The second series comprised 4 monkeys injected with very large quantities of typhus serum (6 to 18 c.c.), 1 with unfiltered, 3 with filtered virus. None reacted to the injection. Subsequently 1 monkey which had been injected with filtered serum and 1 which had been injected with unfiltered serum were reinoculated with virulent blood. Both came down with the disease! Here again, the controls were unsatisfactory and the blood used for the first injection may have been avirulent.

The third series included the injection of a man (one of the co-workers of Nicolle) with 0.25 c.c. of filtered typhus serum. There was no reaction and the control monkey died of some intercurrent disease shortly after inoculation. This series as well leads to no conclusion.

The fourth series is relatively unimportant since the only conclusion to be drawn therefrom is that filtered emulsions of disintegrated leukocytes from virulent blood did not infect a monkey.

The fifth series of experiments¹¹ comprised 2 monkeys injected with filtered blood, which did not react and were not immunized — a typical experiment leading to only one conclusion; namely, that the virus does not pass through the filter.

From Nicolle's work we may conclude that he has presented no evidence that typhus fever virus is filtrable.

To support Nicolle's assumption of the filtrability of the typhus

¹⁰ Bull. 86, Hyg. Lab., Washington, D. C., 1912.

¹¹ Nicolle, Blanc and Conseil, Arch. de l'Inst. Pasteur de Tunis, 1914, 9, p. 84.

virus, the observers mentioned added the single experiment of Wilder,³ who continued the work of Ricketts and Wilder.¹² All their experiments in regard to the filtrability of virus may be classed into 3 series.

The first series comprised 2 monkeys. One monkey, injected with unfiltered blood, died of typhus fever; one monkey injected with filtered blood showed no reaction. The conclusion of Ricketts and Wilder was: "It seems probable, therefore, that the virus of the typhus fever of Mexico may be classed with the unfilterable."

The second series, comprising 2 monkeys, showed the same results. Here, as well, was demonstrated the absence of immunity in the monkey injected with filtered blood mentioned in the first series. The authors' conclusion was: "The evidence is sufficiently strong that the virus of tabardillo [Mexican typhus fever] does not pass through a filter of the type mentioned [Berkefeld]."

Up to this point a definite case for the nonfiltrability of the typhus virus has been made out.

The third series, however, did not confirm the other two. This series completed the second: both the monkey which had been injected with unfiltered blood, and the one injected with filtered blood, were immune. Wilder explains this refractoriness on the part of the monkey injected with filtered blood on the basis that the monkey was naturally immune, or that certain substances passed through the filter which immunized the animal, or that at one stage of the disease the organisms are filtrable.

The only conclusion to be drawn from Wilder's experiment is that the monkey was naturally immune or that avirulent blood was used in the immunity test. The great number of experiments showing that filtered blood neither infects nor immunizes, that a large proportion of monkeys are naturally immune to typhus virus, and the fact that Wilder injected his animal only once, when a second or third injection sometimes is necessary to produce typhus fever—speak against any other conclusion.

Hence, neither of these two observers, Nicolle nor Wilder, has presented sufficient evidence that typhus virus is filtrable.

On the other hand, definite, decisive, and controlled experiments have shown that typhus virus is nonfiltrable. These have been made by Anderson and Goldberger,¹⁰ by Gaviño and Girard,¹³ and by myself.

The classical experiments of Anderson and Goldberger may be

¹² Jour. Am. Med. Assn., 1910, pp. 463, 1373.

¹³ Pub. de l'Inst. Bacteriol. Nationale de México, 1910 (Aug. 23 and Nov. 9) and 1911 (Nov. 12).

grouped into 3 series. The first series comprised 4 monkeys. Two were injected with unfiltered typhus serum; one reacted, while the other did not react to this injection, but did react to a subsequent inoculation. Two monkeys were injected with the same serum filtered; neither reacted, nor were they immunized.

The second series comprised 4 monkeys. The results were identical with those of the first series.

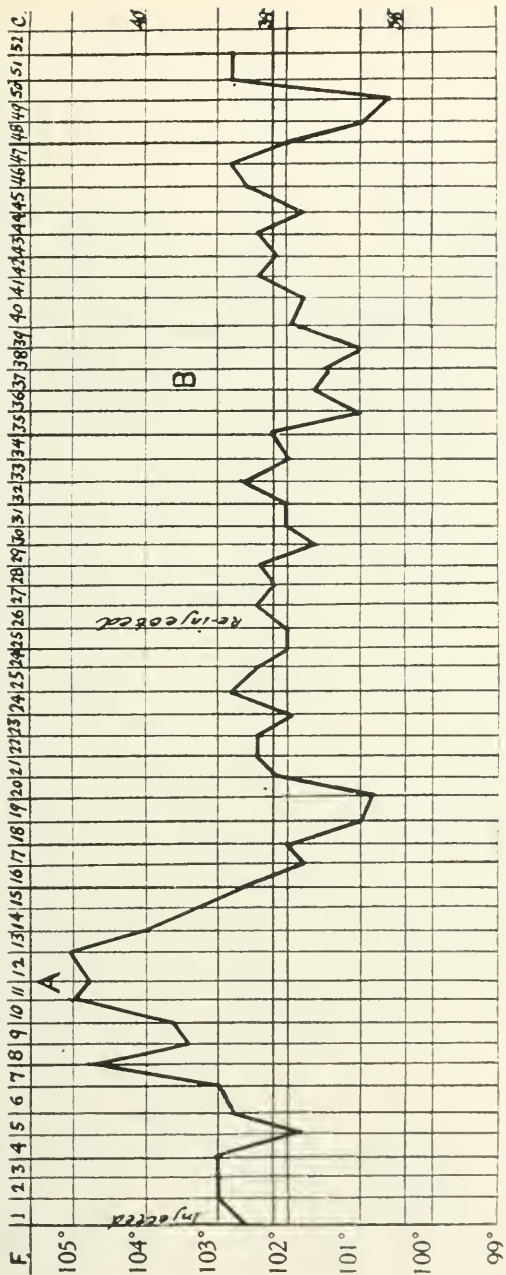
The third series comprised 2 monkeys which were injected with the filtered serum from clotted typhus blood — the blood having been centrifugated. They did not react nor were they immunized. These experiments remove the main objections of Nicolle, who stated that the virus is intraleukocytic and unless clotted whole typhus blood is used after centrifugation (so as to break up some leukocytes), the results may be negative.

From these experiments Anderson and Goldberger conclude: “. . . there is no evidence that the virus in the blood of typhus is able to pass the Berkefeld filter.”

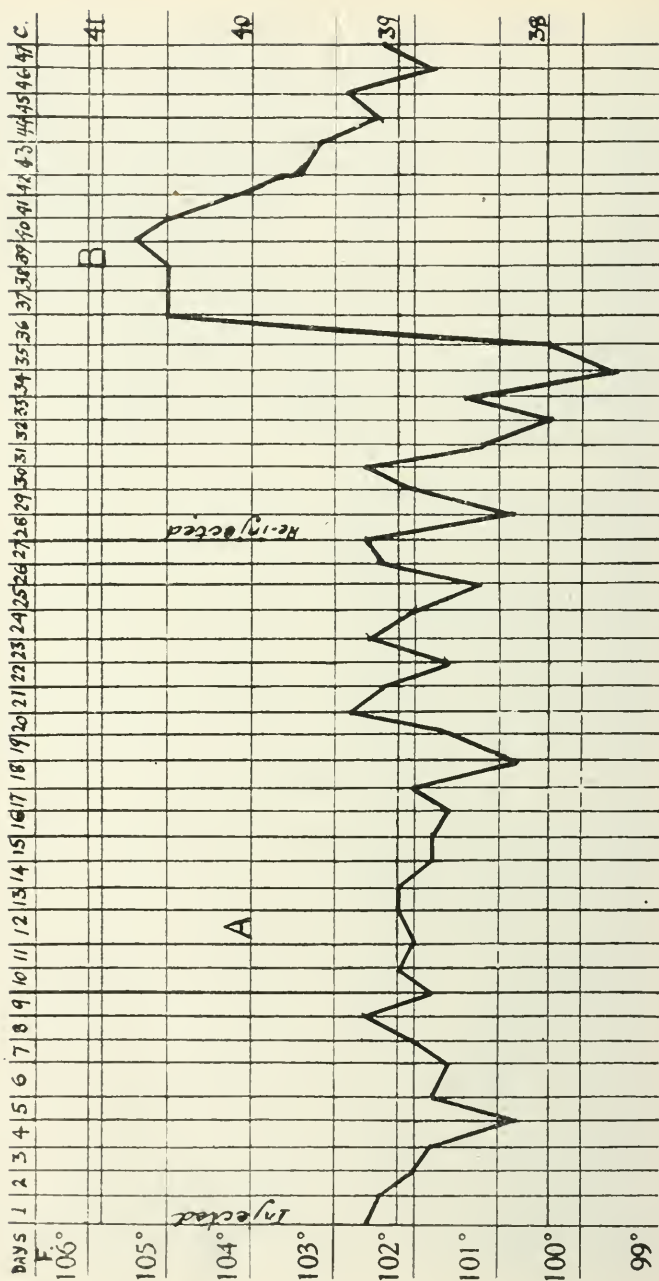
Gaviño and Girard carried out experiments which correspond to the fourth series of Nicolle's work. They injected intraperitoneally into a monkey at the height of its typhus fever, peptone broth. Three hours later the peritoneal fluid, rich in red and white blood cells, was removed. A filtrate of this failed to infect a monkey and, as a result, they conclude that the virus is nonfiltrable.

I wish now to present further evidence demonstrating the non-filtrability of typhus virus — the result of experiments suggested by Dr. Libman. He advised the use of guinea-pigs for this work for the following reasons. While natural immunity against typhus occurs in a great percentage of monkeys, this condition is almost unknown in mature guinea-pigs. Baehr¹ found that when guinea-pigs failed to react to an injection of typhus virus, the refractoriness was not due to the immunity of the animal but rather to a lack of infectivity of the blood. For instance, when a guinea-pig failed to react, all guinea-pigs in the same series, that is, all injected with the same blood, failed to react. Anderson¹⁴ in a series of 210 guinea-pigs found that only 4.7% failed to react to what he regarded as virulent blood. But in all these no subsequent immunity tests were made; all might have reacted to a second or even a third injection of virus. Hence we see that guinea-pigs are peculiarly fitted for experiments on the filtrability of the virus.

¹⁴ Jour. Med. Research, 1914, 25, p. 467.



Temperature-chart 1. A. Reaction of Guinea-pig 545 inoculated with unfiltered blood from Guinea-pig 529. B. No reaction to a subsequent injection of the same virus a demonstration of the immunity conferred by the first inoculation.



Temperature-chart 2. A. Absence of reaction in Guinea-pig 546 inoculated with a filtrate of blood from the same guinea-pig 529 mentioned in Temperature-chart 1. B. Reaction to a subsequent injection of the same virus used for Guinea-pig 545—a demonstration of the absence of immunity after inoculation with the filtered virus.

In my experiments all factors which would tend to interfere with the results were carefully eliminated. The virus employed was one which had been obtained originally from a case of endemic typhus fever and had been transmitted for about 5 years from guinea-pig to guinea-pig. Its incubation period was more or less constant — about 10 days; the duration of the fever was usually from 4 to 6 or 7 days. In each series of experiments, the same blood was used throughout; the unfiltered blood was obtained from the material left on the outer side of the candle; the filtered blood, from what passed through. The filter was of the Berkefeld type, size N, which does not allow the passage of *Bacillus typhosus*, *Micrococcus aureus*, *Bacterium coli*, *Streptococcus anhemolyticus*, or the typhus bacillus. The filters were new and the whole apparatus was sterilized in moist steam. The pressure employed was 500 mm. Hg and the time taken in the filtration was within one-half hour.

The blood was prepared for filtration as follows: A guinea-pig was exsanguinated at the height of a typhus-fever reaction. The whole blood was defibrinated and cleared by centrifugation. The serum (which carries the virus) was diluted with twice its volume of physiologic salt solution to prevent clogging of the filter. For the subsequent immunity test, whole defibrinated blood containing the same strain of virus was used.

Guinea-pig 545 (see Temperature-chart 1) was inoculated intraperitoneally with an amount of unfiltered diluted serum from typhus Guinea-pig 529, equivalent to 2 c.c. of the whole serum. Eleven days later, the animal developed a fever of over 40 C. (104 F.) lasting for 4 days; then the crisis occurred. After 11 days of normal temperature, the animal was reinoculated with virus from a typhus-fever guinea-pig (4 c.c. of defibrinated blood from Guinea-pig 572); there was no reaction, tho 3 c.c. of the same blood gave rise to the fever in a control guinea-pig (No. 577) after an incubation period of 10 days.

In other words, the unfiltered blood gave rise to typhus fever which immunized the animal against a subsequent injection of the virus.

Guinea-pig 546 (see Temperature-chart 2) was inoculated with the filtrate of the same serum used for Guinea-pig 545. An amount equivalent to 4 c.c. of the whole serum was injected intraperitoneally. There was no reaction for 28 days; at the end of this time the animal was inoculated with one-half the amount (2 c.c.) of the same virus (from Guinea-pig 529) used for Guinea-pig 545. After an incubation period of 9 days, the animal developed typhus fever lasting 6 days.

In other words, the filtered virus neither produced the disease nor immunized the animal.

Upon repetition of these experiments the same results were obtained.

CONCLUSIONS

The most favorable animal for the study of the filtrability of typhus blood is the guinea-pig. In the experiments here reported, it has been conclusively demonstrated that the infective agent of typhus fever belongs to the group of nonfiltrable viruses. This agrees with the previous observation that *Bacillus typhi-exanthematici* is nonfiltrable.

THE INTRACUTANEOUS 'TYPHOIDIN' REACTION *

I. THE PREPARATION AND PROPERTIES OF THE ANTIGEN

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Thus far no decision has been reached regarding the value of the 'typhoidin' skin reaction as a means of determining the immunity which develops after typhoid infection or prophylactic inoculation with vaccines.

Gay and his co-workers¹ still adhere to their original conception that the typhoidin or paratyphoidin skin reactions are specific and indicative of an existing immunity against typhoid and allied fevers. Pulay,² and also Kilgore³ in his first paper, have been the only recent supporters of this interpretation of the typhoidin skin reaction. Evidence, however, has gradually been collected by Nichols,⁴ Austrian and Bloomfield,⁵ Kolmer and Berge,⁶ Kilgore,⁷ and others, which would support the view that the skin reaction is either a true anaphylactic phenomenon—the expression of a cutaneous sensitization to typhoid protein—or a nonspecific dermatic response of the human skin to toxic elements contained in the typhoidin liquid or powder. Kolmer and Berge⁶ consider the reaction an indicator of typhoprotein sensitization, but state that "there is not yet sufficient evidence to warrant its acceptance as an index of immunity in typhoid fever." In similar studies made with 'luetin' and 'diphtherin' skin reactions, they were compelled to the conclusion that these skin tests cannot be used to determine in man the existence or absence of a defensive activity against the respective diseases.

Following the discovery of von Pirquet's cutaneous tuberculin test in 1907, analogous investigations were carried out as to the diagnostic value of the typhoidin reaction in typhoid fever. Chantemesse,⁸ Zupnik,⁹ Link,¹⁰ Deehan,¹¹ Floyd and Barker,¹² and Austrian,¹³ reported favorably on the value of the cutaneous and ophthalmic reactions with various kinds of typhoid preparations.

* Received for publication December 4, 1916.

¹ Arch. Int. Med., 1914, 13, p. 471; 14, p. 697.

² Wien. klin. Wchnschr., 1915, 28, p. 1382.

³ Arch. Int. Med., 1916, 17, p. 25.

⁴ Jour. Exper. Med., 1915, 22, p. 780.

⁵ Arch. Int. Med., 1916, 17, p. 663.

⁶ Jour. Immunol., 1916, 1, p. 409.

⁷ Arch. Int. Med., 1917 (still to be published).

⁸ Deutsch. med. Wchnschr., 1907, 33, p. 1572.

⁹ München. med. Wchnschr., 1908, 55, p. 148.

¹⁰ Ibid., p. 730.

¹¹ Univ. of Penn. Med. Bull., 1909-1910, 22, p. 192.

¹² Am. Jour. Med. Sc., 1909, 138, p. 188.

¹³ Bull. Johns Hopkins Hosp., 1912, 23, p. 1.

Kraus,¹⁴ Wolff-Eisner,¹⁵ Goodmann and Sutter,¹⁶ Chauffard and Troisier¹⁷ on the other hand, failed to confirm their statements, and questioned the specificity of the reactions. Kraus, Lusenberger, and Russ,¹⁸ Entz,¹⁹ Rolly,²⁰ and von Szontagh²¹ showed that a high percentage of normal individuals also react to various bacterial toxins, and among them the typhoid toxin. It would appear that very little attention has been paid to all these studies, tho it is quite apparent that the typhoidin powder now used contains most of the substances which, in the observations of Entz, Kraus, and others, must have given rise to nonspecific skin reactions in man. That not all the skin reactions are true anaphylactic or, as in the Schick test, true antitoxin-toxin neutralization phenomena, is pointed out by Besseau and Schwenke²² in a recent report on the local reaction in children to diphtheria broth. They consider the coccostabil endotoxin of the diphtheria bacillus the substance which caused the skin efflorescence in a certain percentage of children injected intracutaneously with neutralized toxin-antitoxin mixtures. In their opinion the irregular susceptibility of man to these substances and the absence of this type of sensitiveness in animals make further investigations desirable, inasmuch as cutaneous tests are now more and more used for diagnostic and experimental purposes.

Our studies on the nature, mechanism, and value of the typhoidin reaction are part of extensive observations on typhoid infection in rabbits.

An opportunity to solve some of the problems presented was afforded in the availability of animals which had been used in the preparation of various antisera and in other immunologic experiments.

An adequate presentation of this subject would require a discussion of the basic principles underlying skin reactions in general and their relation to anaphylaxis, infection, and immunity. In the first of these communications we wish to confine our attention to the methods of preparation and standardization of typhoidin and similar glycerin extracts, leaving a consideration of the other problems to the papers which follow.

METHODS OF PREPARATION OF TYPHOIDIN AND SIMILAR GLYCERIN BROTH-CULTURE EXTRACTS

Little attention has been paid to the exact mode of preparing typhoidin. The literature on this subject states only incompletely the composition and the nature of the peptones used in the medium. In

¹⁴ Wien. klin. Wchnschr., 1907, 20, p. 344.

¹⁵ Die Ophthalm- und Cuti-diagnose der Tuberculose. 1908.

¹⁶ Univ. of Penn. Med. Bull., 1909-1910, 22, p. 81.

¹⁷ Compt. rend Soc. de biol., 1909, 66, p. 519.

¹⁸ Wien. klin. Wchnschr., 1907, 20, p. 1385. München. med. Wchnschr., 1910, 57, p. 437.

¹⁹ Ibid., 1908, 21, p. 379.

²⁰ München. med. Wchnschr., 1911, 58, p. 1285.

²¹ Arch. f. Kinderh., 1912, 58, p. 326.

²² Monatschr. f. Kinderh., 1915, 13, p. 397.

fact, some workers have failed to emphasize one of the most important points underlying its preparation; namely, that the typhoidin thus far used is a glycerin extract of the typhoid bacillus, and that, as is well known from extensive studies on tuberculin, mallein, 'abortin,' and the like, such extracts contain a large amount of salts and other extractives. It is also a well-established fact that broth cultures, or autolysates and similar preparations of pure bacterial protein, not extracted with glycerin, give less distinct and consistent reactions than do glycerin extracts; and yet, for local anaphylactic reactions tuberculin or mallein is more efficacious if it contains elements of the bacterial bodies (Klimmer²³).

For cutaneous or ophthalmic reactions, workers have found it preferable to remove the glycerin and to purify the various tuberculin and mallein extracts by precipitation with alcohol. Since the latter procedure is sometimes attended with difficulties, various other means have been devised to obtain a suitable powder. The procedure followed by us will be discussed in detail in subsequent paragraphs.

It has been customary to consider the typhoidin a preparation analogous to tuberculin, but we feel that such a conception is not fully correct, because the metabolism of the tubercle bacillus and that of the typhoid bacillus differ so widely that it is not unreasonable to assume that their glycerin-broth cultures must contain catabolic and anabolic products differing greatly in character. That this is the case is shown by the experience that concentrated tuberculin remains active for more than one year, but that a similar typhoidin specimen is usually inert after one month. Furthermore, we know nothing about the value of the toxic pyrogenic substances and their gradual disintegration in typhoidin. In the case of mallein, however, one of us²⁴ has found that an old powder, practically free from nonspecific toxic elements, gives very selective and, from a diagnostic viewpoint, better results than do fresh, highly toxic solutions. In order, therefore, to determine the value of typhoidin as an aid in immunologic research, it is of the greatest importance that these points be carefully investigated.

In the present series of experiments we have employed glycerin extracts of broth cultures, reserving for future investigation the value of pure bacterioprotein as an antigen.

²³ Handbuch der Serumtherapie und Serundiagnostik in der Veterinar Medizin, 1911, p. 109.

²⁴ Meyer, Jour. Infect. Dis., 1913, 12, p. 171.

In previous reports on typhoidin we failed to find a reference to the standardization of the product, especially in so far as its antigenic properties are concerned. Furthermore, no explanations are given in the literature to account for the well-known observation that old preparations are inactive. We therefore felt that these and other questions required further study.

General Technic for the Preparation of Typhoidin.—Erlenmeyer flasks containing 500 c.c. of a veal infusion (1% peptone; 0.5% sodium chlorid; 5% glycerin) of a reaction varying from 0.5 to 1 (pH^+ 7.4 to 7.6) were inoculated with one strain of *B. typhosus*. The flasks, loosely capped with tinfoil, were incubated for varying periods at 37 C., and then tested for purity. One-half of the culture was, without previous sterilization, concentrated to one-tenth of its volume on the water-bath at a temperature which rose gradually from 40 C. to 60 C.

The syrupy milky fluid, after it had stood from 24 to 48 hours, was centrifugated at low speed to remove some of the phosphates. Usually no attempt was made to clear it. All the manipulations of concentrating, etc., were done in diffused light or in the dark. The final product was kept in brown bottles in the refrigerator until ready for precipitation.

From 10 to 20 c.c. of the concentrated typhoidin were precipitated in from 200 to 400 c.c. of absolute alcohol. It is important that the alcohol be absolutely water-free and that the syrupy typhoidin be added to the alcohol only by drops. In case the milky mixture does not flocculate in a few minutes, a few sodium chlorid crystals should be added. As soon as the coarse light-yellowish precipitate has settled in the ice-chest, the supernatant alcohol is siphoned off and replaced by fresh absolute alcohol. Then the suspension is quickly centrifugated, the alcohol poured off, and the sediment suspended in a very small amount of ether-alcohol. Finally, the voluminous precipitate is filtered off, collected on fresh sterile filter paper or clay plates, and dried in vacuo over sulfuric acid or fresh calcium chlorid. Several preparations were redissolved in a 1% sodium-chlorid solution and precipitated again. Perfectly white or light-brownish granular powders were finally obtained. Such preparations are slightly, if at all, hygroscopic, but are soluble in salt solution; for example, a portion of the preparation Typhoidin B15 was kept for 4 weeks in a room in which steam from sterilizers added considerably to the high humidity of the foggy climate. The powder did not cake, and remained granular. Moisture was absorbed according to the increased weight of the powder and, together with the effect of the diffused light, was probably responsible for the deterioration and complete loss of antigenic properties of this test sample of typhoidin.

As a rule, these powders dissolved readily in salt solution forming turbid or opalescent solutions or suspensions in which the bacterial debris usually settled in the form of a grayish sediment. The repeated treatment with alcohol made these powders insoluble, however, so that only suspensions could be obtained. It is not unlikely that such preparations, on account of their colloidal nature, act as irritants, and are probably responsible for the nonspecific reactions. To overcome this possible insolubility of the typhoidin powders, we would recommend the filtration of the concentrated extracts, and the careful use of absolute alcohol. One should particularly avoid repeated washings and a too long contact of the preparation with alcohol.

All typhoidin powders were kept in brown bottles, protected from the light in desiccators at room temperature. Only in special experiments were polyvalent glycerin extracts used; in the majority of instances the typhoidin preparations were monovalent. Shortly before use, definite amounts were dissolved in carbolized (0.5%) salt solution. The glycerin broth-culture extracts of various experiments were prepared in the same manner. The broth used for the control powders was incubated with the cultures and handled absolutely in the same manner as that outlined. It was in this connection that we first found that repeated treatment with alcohol produces insoluble powders.

We found also that the composition of the culture medium and its reaction have a considerable influence on the preparations obtained. It will be shown (Experiment-group 2) that a 2% peptone medium or Martin's broth gives a very toxic typhoidin, and that a potato-glycerin-glucose-Lemco-broth* is better than veal broth. The type of peptone also influences the toxicity of the typhoidin; peptone-Chapoteaut gives a somewhat more toxic powder than Witte's peptone. An optimal reaction of the culture fluid was always chosen and was found to be about pH⁺ 7.4 to 7.6, as determined by our colorimetric method.²⁵

It will be shown later that the amount of broth inoculated with the typhoid strains is also of importance in the preparation of a good product. If the broth is kept in a layer of 2 cm., the toxicity is remarkably increased. In small amounts the sterile filtrate is toxic to rabbits on intravenous inoculation, and the number of skin reactions on normal rabbits is strikingly increased. To overcome this difficulty, we grew the bacteria in flasks as completely filled as possible. We have also chosen the glycerin potato broth and prolonged incubation (20 to 30 days) to overcome some of the factors which we believe impart to the typhoidin preparations a high degree of primary toxicity. A more extensive discussion of these important details will be undertaken in connection with the experimental data presented in later paragraphs.

Standardization of Typhoidin and Similar Preparations.—As a result of our experience with abortin, reported in 1913,²⁶ in which we followed the suggestions of Calmette and Massol²⁷ on tuberculin, we have chosen the complement-fixation test as a means of determining the exact amount of specific antigenic substances in a given preparation. Preliminary tests had already indicated that neither the general appearance of the typhoidin nor its method of preparation have much influence on its antigenic properties. On the other hand, it is generally conceived, from some work done with mallein, that the diagnostic value of a preparation depends largely on its antigenic units. This fact will be more fully considered and illustrated by subsequent experiments.

In general the antigenic value of a preparation may be ascertained either by complement-fixation or by inoculation tests.

* Raw potato (500 gm.) in 2000 c.c. of water, is macerated for 12 hours at 60 C., strained, and 10 gm. of meat extract (Lemco), 10 gm. of salt, and 20 gm. of peptone are added, the whole steamed, rendered faintly alkaline to litmus (pH⁺ 7.4) filtered after steaming for 1½ hours, and 1% glucose and 1% glycerin added before distribution in flask.

²⁵ Bull. Johns Hopkins Hosp., 1916, 27, p. 16.

²⁶ Jour. Infect. Dis., 1913, 13, p. 355.

²⁷ Compt. rend. Soc. de biol., 1912, 72, p. 15.

TABLE 1
STANDARDIZATION OF TYPHOIDIN BY MEANS OF COMPLEMENT-FIXATION TESTS

Tube	Typhoidin 17, Diluted 1:100	Rabbit Immune Serum 2×Titer	Complement 1:4	Saline Solution (0.85%)	Antisheep Hemolysin (1:100)	Blood-cell Suspension (1%)	Result
1	0.01	0.001	0.05	1.5	0.2 (twice the hemolytic unit)	0.5	No hemolysis
2	0.0075						
3	0.005						
4	0.0025						
5	0.002						
6	0.001	0.1	0.05	1.5	0.2 (twice the hemolytic unit)	0.5	50% hemolysis
7	0.00075						
8	0.0005						
9							
10	0.01						
11	0.0075	0.001 normal serum	0.05	1.5	0.2 (twice the hemolytic unit)	0.5	Hemolysis
12	0.005						
13	0.0025						
14							
15	0.1						
16	0.05	0.1 normal serum	0.05	1.5	0.2 (twice the hemolytic unit)	0.5	Hemolysis
17	0.01						
18	0.0075						
19	0.005						
20							

The technic of complement-fixation for this particular purpose deviated very little from the method employed in our laboratory and recently described in detail.²⁸ Double amounts of a highly potent rabbit typhoid-immune serum, prepared with the same strains which had been used for the manufacture of the typhoidin, were titrated against descending dilutions of the powder. The powder was usually dissolved in 100-mg. doses in 10 c.c. of carbolized salt solution. An antisheep hemolytic system was used as indicator. In Table 1 a typical determination is given.

Table 1 shows that the smallest amount of typhoidin causing fixation of complement, is 0.002 c.c. of a solution of 0.1 gm. of typhoidin in 10 c.c. of salt solution. Neither nonspecific fixation nor hemolysis was observed in the control tubes of the serum or the antigen with and without normal serum. The amount of 0.002 c.c. was designated by us as an extract-unit (e.u.), and we could easily determine the number of such units per 10 c.c. or for 0.1 gm. of a given typhoidin. For purposes of clarity, the method of deriving the result of the example cited in Table 1 may be given. Thus the proportion would be: 10:0.002, which gives 5000 units per 0.1 gm. or 50 units per 0.001 gm.—the dosage usually employed in our experiments. A preparation containing about 20 to 40 e.u. may be considered a good product. The strength of the preparation gradually diminishes in salt solution, but with the method just outlined it was possible to determine, with little difficulty, the relative quality of the typhoidin preparation chosen for some of the experiments.

In Table 2 some of the unit determinations are classified to illustrate the value of the method as a guide in anaphylactic skin reactions. The results recorded for Nos. 3 and 4 explain the observation that preparations older than 4 to 6 months are poor reagents in animals. That some of the reactions obtained in man with such old typhoidin preparations are doubtless not due to the antigenic substances of the preparations, but to some nonspecific irritant, is made also more plausible by these tests.

²⁸ Jour. Exper. Med., 1916, 24, p. 516. Arch. Int. Med., 1917 (still to be published).

TABLE 2
CLASSIFICATION OF UNIT DETERMINATIONS FOR TYPHOIDIN

No	Preparation	Appearance	Smallest Dose which Completely Fixes the Anti-serum	Extract Units per 0.001 gm.
1	Typhoidin 3. Veal infusion + 1% Chateaut-peptone. Strain Olsen, fresh	Coarse light-brownish powder, readily soluble	0.001	19
2	Typhoidin 3, dissolved in carbolized salt solution 1:100 and kept at ice-chest temperature for 22 days	Light-brownish fluid with slight grayish sediment	0.005	26
3	Typhoidin 2. Veal infusion + 1% Chateaut-peptone. Strain Dorset. Re-precipitated Dec. 15, 1915	Light whitish powder, readily soluble	0.003	33.3
4	Typhoidin 2; freshly dissolved powder June 7, 1916	Light-yellow turbid fluid	0.077	1.3
5	Typhoidin 4. Veal infusion + 1% Witte's peptone. Strain Kuhn. Incubation 18 hours, fresh	Slightly caked, but readily soluble, powder; light-brown	0.01	10
6	Typhoidin 4, 2 months later	Same powder	0.025	4
7	Typhoidin 17 (Olsen). Potato glycerin broth fresh on March 28, 1916	Finely granular deep-brownish powder, soluble with a considerable grayish sediment	0.002	50
8	Typhoidin 17. June 7, 1916	Fresh solution	0.003	33.3
9	Martin's-broth typhoidin (Olsen)	Fine powder, easily soluble to an opalescent fluid	0.005	10
10	Distilled residue of wash alcohol of Typhoidin B	Smeary gummy substance, soluble in salt solution	0.1	1

It is well to bear another important point in mind, namely, that repeated resolution and reprecipitation of the typhoidin produces a preparation poor in extract units, the residue of the wash alcohol after distillation giving, when dissolved in salt solution, fixation of the complement.

From our tests it is not possible to state with certainty that the latter is due to protein substances or to the lipid-like alcohol-soluble material of the typhoidin. Whenever possible, the antigenic properties of other bacterial extracts were tested by complement-fixation, but our main experience for the most part is based on the work with typhoidin.

Finally, the antigenic value of some of the typhoidin preparations was also tested by direct intravenous injections of rabbits with 0.1 to 0.01 gm. of powder dissolved in salt solution. The results with 3 typhoidin preparations are shown in Chart 1.

As a rule, recently isolated strains of *B. typhosus* give a typhoidin poor in antigenic properties (Curve 3). Repeated purification reduces somewhat the agglutinin-stimulating effect of the typhoidin powder (Curve 2).

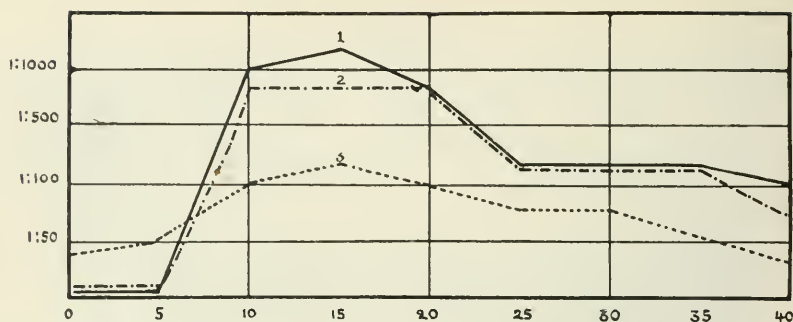


Chart 1. The average agglutination titers of 6 rabbits inoculated with 0.01 gm. of typhoidin.

— 5-day-old culture of an old strain, not filtered or purified.
 - - - 5-day-old culture of the same strain, purified by 3 successive precipitations.
 5-day-old culture of a recently isolated strain, not purified.

MODE OF APPLICATION OF THE GLYCERIN EXTRACTS

All tests were carried out by the intracutaneous inoculation of 0.001 to 0.0001 gm. of the powder dissolved in carbolyzed salt solution. The dilutions of the typhoidin preparations were so chosen that 0.1 c.c. contained the amounts of antigenic substance stated. Only fresh solutions or suspensions of the powder were used. The skin of the back was shaved from 2 to 3 days previous to the tests, and was carefully freed from the irritant soap and cleansed with alcohol before the injections were made. Platinum-iridium needles and Record syringes were used throughout the tests. Some experience, to be sure, is required in applying the suspensions intracutaneously, but we cannot agree with Kolmer²⁹ that the intracutaneous applications of test fluids is any more difficult in the rabbit than in the guinea-pig or any other laboratory animal.

Ordinarily 2, sometimes from 3 to 4, different extracts in various dilutions were tested on the same animal. Each preparation was compared with its control powder made from the sterile culture medium. Naturally, sites of injection were always chosen as far apart as possible so as to avoid fusion of the reactions. In this we were successful in the majority of experiments, but in some the toxicity of the preparations resulted in such a marked edema that complete separation of the reaction areas was not possible.

REACTIONS CAUSED BY THE EXTRACTS

It is perhaps not generally known that rabbits are not very suitable for skin tests. It may be well to recall the fact that tuberculous animals of this species react slightly, if at all, to the ordinary tuberculin test, but fairly well to the intracutaneous or subcutaneous application of a suspension of dead tubercle bacilli (Klimmer³⁰). Experience with this reaction has taught us that a positive result is characterized by a red areola surrounding the needle puncture, with marked

²⁹ Jour. Amer. Med. Assn., 1916, 67, p. 719.

³⁰ Handbuch d. Serumtherapie, etc., 1911, p. 120.

swelling, induration, and increased heat at the reacting area—these features becoming more striking usually within 24 hours, and frequently lasting from 2 to 3 days.

Similarly, a specific reaction with typhoidin is, in our experience, characterized by an area of erythema and a central indurated papule. The diameter of the areola may vary from 1 to 3 cm., depending on the concentration of the antigenic extract and the time interval after the injection. Under ordinary conditions the maximum of this reaction is reached in about 24 to 36 hours after the injection. If at the end of this period the longest diameter of the areola measures 10 mm. or more, the reaction may be regarded as positive. A positive reaction persists to the 40th hour and begins to disappear very gradually, so that at about the 72nd hour only faint outlines of the areola and papule can be noticed. As a rule, the reading at the end of 24 hours shows a greater intensity of reaction than after 48 hours. Sometimes delayed reactions reach their height only at or after 48 hours. This makes readings after 72 hours essential. A response—probably not due to an antigen-antibody reaction—appears more rapidly in the first 24 hours and fades with equal rapidity. The diameter of the areola may be the same as in a positive reaction, but the edema and hyperemia are less marked, and particularly the papule-formation is, for the most part, absent. Such reactions have been recorded in the experimental data as "red areas." The control extracts usually cause a small areola of a maximal diameter of 0.5 cm., and in these instances only a reddish spot with very little induration is noticed. These are referred to as "red spots," or, whenever smaller, as "needle puncture." In a few instances very marked edema and infiltration of the skin with little or no hyperemia were observed in control animals. Such reactions are difficult to explain, but in our experience they are found only when the preparations are rich in colloidal substances, or they may be the result of faulty technic. The yellowish center in the papule, or nodule, develops only when highly concentrated extract solutions are used, or if the needle puncture has become infected with skin organisms, or if the test powder has become contaminated.

The selection of typhoidin dilutions gave us an opportunity to work quantitatively and, in numerous instances, this was of extreme value in forming an opinion as to the specificity of a reaction. When a preparation was found to be very irritant to the rabbit in high concentrations, as was apparent from the reaction it produced in normal animals,

a dilution was then chosen which gave a negative result. Such dilutions gave, as a rule, well-marked reactions in sensitized or immune animals, and the intensity of the areola persisted usually for at least 48 hours. Furthermore, the difference in the diameters of the indurated areolae of the various dilutions in comparison with the control spot were usually so striking that they assisted greatly in forming judgments concerning positive or negative reactions. For similar reasons we tested usually from 3 to 4 different preparations and their corresponding controls, on the same animal.

For the typhoidin test it was not difficult, in most instances, to interpret the reactions obtained in the immunized animals; the difference between the reactions caused by the typhoidin, or other extracts, and those produced by the control powders, was always sufficiently well marked. This observation is in striking contrast to the well-established fact that the human skin very frequently reacts just as intensively to the control powder as to the typhoidin and, therefore, presents extreme difficulties in the interpretation of the reactions (Kolmer⁶).

To obtain comparative data the diameters of the indurated areolae were measured with a machinist's vernier gauge at intervals of 16, 24, 48, and 72 hours after the intracutaneous injections. In the records of the experiments the figures tabulated represent the carefully measured diameters of the areolae expressed in centimeters.

ANIMAL EXPERIMENTS

The various intracutaneous tests with bacterial glycerin extracts were made on about 100 immunized, and 30 normal rabbits.

Immunization.—The method of immunization varied according to the antigens used. A discussion of this point will therefore be deferred until the separate experiments are considered. At least one or more months elapsed before the animals were tested. In a few cases an injection of living organisms was given between the 1st and 2nd test. This was done to test the resistance of these animals, as well as with a view to producing carriers. Some animals were extensively immunized with living and dead bacteria; in a few cases as many as 17 injections were given.

The degree of immunity was observed in the appearance and persistence of agglutinins and complement-fixing antibodies, a large number of animals being tested regularly at 10-day intervals. The sera were collected a day previous to the application of the allergic skin tests and the necessary tests were performed within the next 48 hours.

Agglutination Tests.—The macroscopic technic with standardized, formalized, or carbolized broth cultures was used. Each rabbit serum was tested

with the strain of *B. typhosus* with which it had been immunized. The control animals and those treated with bacteria other than typhoid were tested either with their respective organisms, or with the typhoid strain used in the typhoidin preparation.

Complement-Fixation Tests.—Complement-fixation tests were conducted with an antishoop hemolytic system and the corresponding broth-culture antigens. To these, references have been made in previous communications.²⁸ Heated and carbolized 18-hour-old Liebig's-broth cultures were used, for the most part, in case of the typhoid bacillus and allied organisms. Suspensions or aqueous extracts were employed in a few instances only. The antigens were used in amounts corresponding to one-fourth to one-third of their anticomplementary units.

Preliminary experiments on rabbits had demonstrated that the concentration of the typhoidin powder suspended or dissolved in carbolized salt solution influenced, as might be expected, the degree of reaction. A series of experiments was planned, therefore, to determine the amount of typhoidin necessary to produce characteristic reactions in highly immune animals. Furthermore, to test the specificity of the reactions, an extract of the fowl-typhoid bacillus was used as a control. Theobald Smith and TenBroeck have recently shown the close relationship of the bacillus of fowl typhoid and *B. pullorum* to the human-typhoid bacillus, and our studies along similar lines have confirmed their statements, namely, that the fowl-typhoid bacillus in broth media produces a strong toxin for rabbits. It was therefore of interest to determine whether the rabbit skin is sensitive to these nonspecific substances and whether the reactions obtained with them are distinguishable from the specific skin reactions obtained with typhoidin.

Some of these results are discussed in experiments of Groups 1 and 2.

EXPERIMENT-GROUP 1

Twenty-one rabbits were immunized by various methods. The following organisms were used: *B. typhosus*; the fowl-typhoid bacillus; *B. pullorum*; and *B. dysenteriae*. Four normal animals served as controls. These were tested on Jan. 31, 1916, with a glycerin-extract typhoidin and an identical preparation produced with the fowl-typhoid bacillus.

Nine rabbits (Nos. 314, 317, 319, 321, 323, 324, 325, 326, 333) were intensely immunized against various recently isolated strains of *B. typhosus* (for most of which we are indebted to Dr. F. P. Gay); 7 injections, at weekly intervals, were given intravenously until Nov. 8, 1915. Judged by serum reactions, most of the animals still showed, 84 days after the last injection, a high degree of immunity. Three rabbits (317, 319, 321) were reinoculated 9 days previous to the test, with one-half blood-agar slant, of the same typhoid strain with which they had been immunized.

Four rabbits (339, 340, 341, 576) were immunized with 3 doses of Army vaccine (obtained through the courtesy of Major H. J. Nichols, of the U. S. Army

TABLE 3
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Animal	Immunization	Last Injection (1915)	Serologic Tests Jan. 27, 1916		Hours	Typhoidin 3	
			Agglutination	Complement-Fixation		1:100	1:500
314	B. typhosus Rawlings, 7 injections	Nov. 8	1:2000	>0.003	18	(cm.) 2 x 2, W. D.	(cm.) 1.6 x 1.6, W. D.
					28	1.6 x 1.6	1.2 x 1.2
					54	0.5 x 0.5, nodule	N. P.
323	B. typhosus 16, 7 injections	Nov. 8	1:800	>0.01	18	1.9 x 1.9, D. O.	0.2 x 0.2
					28	1.8 x 1.8	Sl. red spot
					54	0.4 x 0.4	N. P.
324	B. typhosus 17, 7 injections	Nov. 8	1:2000	0.003	18	1.9 x 1.9, W. D.	N. P.
					28	2.1 x 2.1, W. D.	N. P.
					54	0.5 x 0.5	N. P.
325	B. typhosus 18, 7 injections	Nov. 8	1:800	>0.01	18	2.2 x 2.2, W. D.	1.0 x 1.9, W. D.
					28	2.2 x 2.2, W. D.	1.7 x 1.7, W. D.
					54	1.3 x 1.3, W. D.	0.5 x 0.5, nodule
326	B. typhosus 19, 7 injections	Nov. 8	1:1000	>0.01	18	1.8 x 1.8, W. D.	1.3 x 1.3, R. I.
					28	1.3 x 1.3, W. D.	0.8 x 0.8, W. D.
					54	0.1 x 0.1	N. P.
333	B. typhosus 47, 7 injections	Nov. 8	1:1000	0.005	18	2 x 2, R. I.	0.8 x 0.8, R. I.
					28	1.8 x 1.8, R. I.	0.2 x 0.2
					54	N. P.	N. P.
339	Vaccine A (usual strength)	Nov. 30, 48 days after	1:2000	<0.003	18	1.8 x 1.8, W. D.	1.3 x 1.3, D. O.
					28	1.8 x 1.8, W. D.	0.7 x 0.7
					54	1.5 x 1.5, W. D.	N. P.
340	Vaccine A	Nov. 30, 48 days after	1:4000	0.005	18	2.4 x 2.4	1.4 x 1.4
					28	2.2 x 2.2	1.1 x 1.1
					54	N. P.	N. P.
341	Vaccine A	Nov. 30	1:4000	0.005	18	2.2 x 2.2, W. D.	0.9 x 0.9
					28	1.9 x 1.9, W. D.	0.5 x 0.5, R. I.
					54	1.3 x 1.3, S. I.	N. P.

KEY TO TABLES

W. D. = well-defined areola
R. I. = deep purplish, indurated area
S. I. = very slight induration
D. O. = diffuse edema
S. O. = slight edema

TABLE 3.—(Continued)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Typhoidin 3	Fowl-Typhoidin 3		Control Powder 3	Remarks
1:1000	1:100	1:1000		
(cm.) 0.3 x 0.3, R. I. N. P. N. P.	(cm.) 1.9 x 1.9 1.8 x 1.8 0.2 x 0.2, nodule	(cm.) 1.4 x 1.4, R. I. 0.5 x 0.5 N. P.	(cm.) 0.1 x 0.1, S. O. N. P. N. P.	Reaction very distinct Re-injected April 25, 1916 Not a carrier
N. P.	D. O.	N. P.	N. P.	Re-injected April 25, 1916 Not a carrier
N. P. N. P.	0.7 x 0.7 0.1 x 0.1, R. I.	N. P. N. P.	N. P. N. P.	
N. P.	0.6 x 0.6	N. P.	N. P.	Re-injected April 19, 1916 Carrier
N. P.	0.2 x 0.2	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	
1.6 x 1.6 1.6 x 1.6 N. P.	0.5 x 0.5, R. I. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	Re-injected April 25, 1916 Carrier
1.8 x 1.8, diffuse R. I. 0.5 x 0.5, R. I. N. P.	0.2 x 0.2 0.2 x 0.2 N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	Re-injected April 27, 1916 Carrier
0.2 x 0.2, R. I. 0.2 x 0.2, R. I. N. P.	0.4 x 0.4 0.1 x 0.1 N. P.	N. P. N. P. N. P.	N. P. N. P. N. = P.	Re-injected April 25, 1916 Not a carrier
D. R. A. 1 x 1, red Red spot	1.3 x 1.3 1.6 x 1.6 Red spot	1.1 x 1.1 0.2 x 0.2 Red spot	N. P. N. P. N. P.	
D. O. N. P. N. P.	2.1 x 2.1 1.8 x 1.8 N. P.	D. O. 0.2 x 0.2 N. P.	N. P. N. P. N. P.	
Sl. red spot Red spot N. P.	1.6 x 1.6, W. D. 1.4 x 1.4 N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	

KEY TO TABLES—Continued

D. R. I. = diffusely indurated and red
D. R. area or D. R. A. = diffuse red blush
S. spot = small spot
N. P. = needle puncture, traumatic reaction
I. R. = indurated red area
D. I. = diffuse induration

TABLE 3.—(Continued)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

Animal	Immunization	Last Injection (1915)	Serologic Tests Jan. 27, 1916		Hours	Typhoidin 3	
			Agglutination	Complement-Fixation		1:100	1:500
342	Vaccine B	Nov. 16, 64 days	1:800	0.05	18 28 54	D. O. 1.9 x 1.9, R. I. Red area	1.2 x 1.2 Red spot N. P.
343	Vaccine B	Nov. 16	1:2000	0.05	18 28 54	(cm.) 1.8 x 1.8, R. I. 1.1 x 1.1, R. I. N. P.	(cm.)
344	Vaccine B	Nov. 16	1:1000	0.05	18 28 54	1.4 x 1.4 1.2 x 1.8 Red area 0.2	1 x 1 0.5 x 0.5 N. P.
346	Vaccine C	Nov. 30	1:2000	0.005	18 28 54	1.5 x 1.5 1.8 x 1.8 0.4 x 0.4	1.2 x 1.2 0.8 x 0.8, W. D. Small red area 0.3
347	Vaccine C	Nov. 30	1:1000	0.005	18 28 54	2.2 x 2.2, W. D. 1.8 x 1.8 0.6 x 0.6	
348	Vaccine D	Nov. 30	1:1000	0.005	18 28 54	1.8 x 1.8, W. D. 2 x 2, indurated N. P.	
349	Vaccine D	Nov. 30	1:1000	0.005	18 28 54	1.5 x 1.5, W. D. 1.5 x 1.5 N. P.	1.5 x 1.5, S. I. 1.5 x 1.5, S. I. N. P.
350	Vaccine D	Nov. 30	1:800	>0.005	18 28 54	2.2 x 2.2, 2 x 2 0.6 x 0.6	0.2 x 0.2, N. P. N. P.
449	Control		1:40	0.1	18 28 54	0.2 x 0.2 N. P. N. P.	N. P. N. P. N. P.
585	Control		1:10	0.05	18 28 54	0.6 x 0.6, R. I. 0.1 x 0.1 N. P.	N. P. N. P. N. P.
317	B. typhosus Rawlings, 7 injections	Nov. 8	1:20,000	0.0005	18 48	1.1 x 1.1, yellow center 1.3 x 1.3, I. R.	1.1 x 1.2, R. I. Red spot
319	B. typhosus Olsen, 7 injections	Nov. 8	1:10,000	0.0001	18 48	1.6 x 1.6, W. D. 1.3 x 1.3, I. R.	1.6 x 1.6, I. R. 1.4 x 1.4, I. R.

TABLE 3.—(Continued)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

Typhoidin 3		Fowl-Typhoidin 3		Control Powder 3	Remarks
1:1000	1:100	1:1000			
Red spot	D. O.	N. P.	0.2 x 0.2		
Red spot	1.5 x 1.5	N. P.	N. P.		
N. P.	N. P.	N. P.	N. P.		
(cm.)	(cm.)	(cm.)	(cm.)		
1.2 x 1.2	1.2 x 1.2	N. P.	N. P.		
D. R. A.	1.1 x 1.1,	N. P.	N. P.		
N. P.	R. I.	N. P.	N. P.		
N. P.	N. P.	N. P.	N. P.		
N. P.	1 x 1	0.1 x 0.1	0.2 x 0.1		
N. P.	0.2 x 0.2	N. P.	N. P.		
Edema	N. P.	N. P.	N. P.		
0.2 x 0.3	1.8 x 1.8	N. P.	N. P.		
0.2 x 0.2	1.6 x 1.6,	N. P.	N. P.		
N. P.	R. I.	N. P.	N. P.		
	N. P.	N. P.	N. P.		
1.5 x 1.5	1.5 x 1.5	0.3 x 0.5	N. P.		
0.6 x 0.6	1.5 x 1.5	0.2 x 0.1	N. P.		
N. P.	N. P.	N. P.	N. P.		
0.9 x 0.9	1.5 x 1.5,	N. P.	N. P.		
1 x 1	red	N. P.	N. P.		
N. P.	1.6 x 1.6,	N. P.	N. P.		
	red	N. P.	N. P.		
	N. P.	N. P.	N. P.		
0.8 x 0.8	1 x 1	0.2 x 0.2	0.2 x 0.2		
0.2 x 0.2	0.5 x 0.5	0.2 x 0.2	0.2 x 0.2		
N. P.	N. P.	N. P.	N. P.		
N. P.	0.2 x 0.3	N. P.	N. P.		
N. P.	N. P.	N. P.	N. P.		
N. P.	N. P.	N. P.	N. P.		
N. P.	0.9 x 1	N. P.	N. P.		
N. P.	0.1 x 0.1	N. P.	N. P.		
N. P.	N. P.	N. P.	N. P.		
N. P.	N. P.	N. P.	N. P.		
N. P.	N. P.	N. P.	N. P.		
N. P.	N. P.	N. P.	N. P.		
	Fowl-Typhoidin 3, 1:10			Control Powder 3	
D. R. A.	1.1 x 1.2	1.4 x 1.4, not raised,	1.8 x 1.8, R. I.	N. P.	Re-injected Jan. 25, 1916 Not a carrier
N. P.	Red spot	N. P.	2.2 x 2.2 R. I.	N. P.	
1.6 x 1.6	1.8 x 1.8	N. P.	2.2 x 2.2	N. P.	Re-injected Jan. 25, 1916 Carrier
1.6 x 1.6, I. R.	1 x 1 I. R.	N. P.	1.5 x 1.5, R. I.	N. P.	

TABLE 3.—(Continued)
 RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

Animal	Immunization	Last Injection (1915)	Serologic Tests Jan. 27, 1916		Hours	Typhoidin 3	
			Agglutination	Complement-Fixation		1:100	1:500
321	B. typhosus Dorset, 7 injections	Nov. 8	1:20,000	0.0005	18	1.5 x 1.5, W. D.	1.5 x 1.5, diffuse l. R.
					48	1.6 x 1.6, I. R.	1 x 1, °I. R.
407	Bacillus of fowl typhoid, Strain Smith, 7 injections	Dec. 16	1:200	>0.003	18	N. P.	N. P.
					26	N. P.	N. P.
					48	N. P.	N. P.
411	B. pullorum, 18 injections	Sept. 22	1:100	0.1	18	2.4 x 2.4, I. R.	D. O.
					48	1.6 x 1.6, I. R.	Red area
427	Bacillus of fowl typhoid, D 4, 17 injections	Sept. 22	1:200	<0.01	18	(cm.) 2.2 x 2.2, W. D.	(cm.) 0.8 x 0.8
					26	1.5 x 1.8, W. D.	0.2 x 0.2
					48	1.7 x 1.7	N. P.
491	B. dysenteriae Mt. Desert (living), 11 injections	Nov. 14	1:50	0	18	1.8 x 1.8	1.1 x 1.1, R. I.
					26	1.9 x 1.9, R. I., W. D.	1.2 x 1.2, R. I.
					48	N. P.	N. P.
556	Control		1:20	0.1	18	N. P.	N. P.
					48	0.2 x 0.4	Edema
					72	N. P.	Edema
568	Bacillus of fowl typhoid A. P. (living), 6 injections	Dec. 16	>1:100	>0.005	18	0.5 x 0.5 R. I.	N. P.
					26	1 x 1, R. I.	N. P.
					48	Small nodule with yellow center	Edema
576	Army vaccine, 3 injections	Dec. 13	1:80	0.05	18	1.6 x 1.6, W. D.	N. P.
					48	1.1 x 1.1, W. D.	N. P.
598	Control		1:20	0.05	18	1.2 x 1.2, R. I.	N. P.
					48	0.2 x 0.2	N. P.
					72	N. P.	N. P.

Medical Corps) at 10-day intervals. The number of organisms employed was the same as was generally used for the prophylactic immunization of man. Forty-seven and 62 days, respectively, after the last injection, the serum of these animals gave decided agglutination and complement-fixation.

Three rabbits (342, 343, 344) were immunized on alternate days with sensitized vaccine sediment. One cubic centimeter of the regular preparation of the California state board of health was given intravenously. The sera of these showed, 76 days after the last injection, some complement-fixation with a

TABLE 3.—(Continued)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

Typhoidin 3	Fowl-Typhoidin 3			Control Powder 3	Remarks
	1:1000	1:100	1:1000 1:10		
1.5 x 1.5, indurated N. P.	1.8 x 1.8, R. I. N. P.	N. P. N. P.	1.6 x 1.6, W. D.	N. P.	Re-injected Jan. 25, 1916
N. P. N. P. N. P.	N. P. N. P. 0.2 x 0.2	N. P. N. P. N. P.	1 x 1	N. P.	
N. P. N. P.	Red area with edema N. P.	N. P. N. P.	0.2 x 0.2	N. P. 0.3 x 0.5	
(cm.) 1.6 x 1.6 0.9 x 0.6, D. R. A. N. P.	(cm.) 2 x 2, R. I. 2 x 2, R. I. 1.9 x 1.9	(cm.) 1.5 x 1.5 1.8 x 1.8, R. I. 0.2 x 0.5, R. I.	(cm.)	(cm.) 0.3 x 0.3 N. P. N. P.	Died on re-injection
1.2 x 1.2, R. I. 0.8 x 0.8 N. P.	1 x 1 1 x 1 N. P.	N. P. N. P.		N. P. N. P. N. P.	
N. P. N. P. Edema	0.6 x 0.6, Red area 0.3 x 0.3, Red area N. P.	N. P. N. P.	1.5 x 1.5, R. I.	N. P. Edema N. P.	
N. P. N. P. Edema	0.2 x 0.2, N. P. N. P.	N. P. N. P. Edema		N. P. N. P. N. P.	On re-injection did not become a car- rier
N. P. 1.1 x 1.1, R. I.	0.8 x 0.2, R. I. N. P.	N. P. N. P.		N. P. N. P.	Rabbit anemic
N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.		N. P. N. P. N. P.	

polyvalent typhoid antigen, and probably on account of repeated bleedings or incomplete sensitization of the vaccine, a fairly high agglutination titer.

Five rabbits (346, 347, 348, 349, 350) were injected with commercial typhoid vaccines. Two animals (346 and 347) were treated with a polyvalent typhoid-paratyphoid vaccine, and 3 rabbits (348, 349, 350) with a monovalent vaccine of the Army type. All the animals were injected at intervals of 10 days and exhibited, 62 days after the last injection, a fair degree of immunity, as judged from the serum reactions.

Five rabbits (407, 411, 427, 491, 568) were immunized over a long period with various dead and living fowl-typhoid organisms, with *B. pullorum*, and with *B. dysenteriae* (Mt. Desert). Some animals (411, 427 and 491) received between 10 and 18 injections, and produced highly potent sera for agglutination tests. One hundred, 31, and 47 days, respectively, after the last injection their sera contained moderate amounts of agglutinins and complement-fixing antibodies.

The four rabbits (449, 556, 585, 598), serving as controls, were of about the same weight and age as the majority of the immunized animals. None of these animals gave serum reactions indicative of an existing immunity against *B. typhosus* and fowl typhoid.

The glycerin extract preparations were both made with veal-Chapoteau glycerin broth in deep layers. Typhoidin 3 was monovalent (Strain Olsen, carrier) and had an antigenic value of 100 e.u. per 0.001 gm. The fowl typhoidin, No. 3, was polyvalent (5 strains: B₃, AP, D, B₂, and Smith); 50 c.c. of the culture of each strain were mixed and then concentrated, as usual. The final powder had an antigenic value of 50 e.u. per 0.001 gm. The results of this experiment are summarized in Table 3.

Most of the rabbits immunized against typhoid reacted with 0.001 gm. of typhoidin, whereas 0.0002 and 0.0001 gm. caused slight reaction or none at all. The reactions were well defined with 0.001 gm., the areola becoming well indurated and remaining so for at least 40 hours. In comparison with the controls, an areola over 1 cm. in diameter was considered as positive. Further evidence that the reaction probably resulted from an antigen-antibody combination, is indicated by the observation that Rabbits 314, 317, 319, 321, 325, 326, 339, 340, 342, 349, which gave marked areolae with 0.001 gm., showed similar intensive reactions in the first 28 hours; this was true, at least, with 0.002 gm. of typhoidin, some of these animals reacting also to 0.0001 gm. of the glycerin extract. With the latter amount, which was absolutely inert for normal rabbits, the degree of induration was different from that obtained with higher concentrations of typhoidin. In most instances only a red blush was present, the method and degree of immunization having apparently very little influence on the intensity of the reaction. An existing typhoid infection was indicated by positive reactions with 0.0002 gm. of typhoidin in Rabbits 319 and 321 in contrast with Rabbit 317, which was only highly immunized. We will discuss this important observation later, more in detail.

It would appear, therefore, that there is no constant relation between the intensity of the skin reactions and the agglutination titer or complement-fixing power of the immune sera of these animals.

The response of the rabbits immunized with organisms allied to the typhoid bacillus is, in many respects, interesting. Three (411, 427, 491) reacted intensively with high, as well as with low, concentrations

of the powder. Apparently those animals which had received the largest number of injections (411 and 427) developed areolae which even surpassed in diameter those of the typhoid-immune rabbits. Immunization with *B. dysenteriae* (Mt. Desert), an organism immunologically different from *B. typhosus*, also produced cutaneous hypersensitiveness to typhoidin preparations. This observation was later confirmed on many other animals and will be the subject of a separate paper. Normal rabbits showed a small areola with 0.001 gm. of typhoidin; only Rabbit 598 developed a small, slightly indurated red area, which disappeared, however, in the first 36 hours. The typhoidin was 49 days old, but apparently contained toxic irritating substances.

In working quantitatively, this nonspecific factor, which we believe is either due to split products or to a high salt concentration, does not need further consideration because the powder was inert on normal rabbits in quantities of 0.0002 gm. and less. The experiments of Group 1 show, furthermore, that individual differences exist in normal rabbits—as far as the inflammatory reactions are concerned, which may result from some of the elements in the precipitated glycerin extracts of *B. typhosus*. The nature and meaning of this inflammatory reaction and the factors which govern the same are just as important a problem for investigation as is the ability of the skin of the infected or immune animal to react in a specific manner.

Fowl-typhoidin 3, which was used in dilution of 1:100 and 1:1000, gave reactions similar in intensity to those with typhoidin. Rabbits 407, 427, and 568, which served to control the value of this preparation, reacted as with typhoidin. An intense reaction was found only in Rabbit 427; of the other two animals, 568 gave a slight reaction, with a very concentrated suspension (0.01 gm.). Rabbit 411, immunized with *B. pullorum*—an organism nearly identical with the fowl-typhoid bacillus—failed to react, and yet the same animal reacted apparently in a specific manner to typhoidin.

Rabbits 323, 324, 325, 326, and 333, immunized with at least 7 injections of one-tenth of an agar slant of various typhoid strains, gave slight or negative reactions. On the other hand, Rabbit 314, which had been immunized with the 'Rawlings' strain, and which had received, on account of the profuse growth of this organism, considerably more bacterial proteins than the other rabbits, as well as Rabbits 317, 319, and 321, which had been inoculated 9 days previously with one-half slant of living typhoid organisms, gave good reactions. The carriers, 319 and 321, reacted slightly better than the hyperimmune

rabbits. The diameter of the areola produced by typhoidin in dilution of 1:500 was about the same as of that produced by fowl typhoidin in dilution of 1:100.

The rabbits treated with various vaccine preparations responded well to the intradermal application of fowl typhoidin. The reaction was, in most of the animals, surprisingly similar in intensity to that to the same concentration of typhoidin. However, the positive reaction is apparently not an indication of a high degree of immunity, because on subsequent injections with twice the lethal dose of living fowl-typhoid organisms, several of the animals (340, 346, 349) which gave very marked and well-defined indurated areolae succumbed to the infection. This important observation has been made the subject of subsequent experiments, and will be discussed later.

It has been demonstrated in observations in Group 1 that the dose of 0.001 gm. of typhoidin powder, or 0.1 of a suspension or solution of this preparation in salt solution, produces skin reactions in immune rabbits which are distinct and can be considered as specific in nature.

Animals which had been immunized recently, and those which had been injected with large doses of bacterial protein, gave more intensive reactions than those which had been treated a long time previously and with smaller doses of bacterial preparations. These observations support our contention that such reactions are the result of cutaneous hypersensitiveness due to bacterial protein. That this sensitization is not strictly specific is already indicated by the group or co-reactions which were noted with fowl typhoidin.

We failed to record noteworthy nonspecific reactions in the control animals and concluded, therefore, that fowl typhoidin or typhoidin does not contain sufficient toxic elements to act as irritants on the skin of rabbits. An experiment was therefore planned in which the most favorable conditions might exist for the production of typhoid and fowl-typhoid toxins. Glycerin broth cultures with high peptone content and free exposure to oxygen were tested on a series of rabbits.

EXPERIMENT-GROUP 2

Eleven rabbits were chosen for this experiment. Three (619, 642, 644) had been injected once with a recently isolated culture of *B. typhosus*. One rabbit (644) had a fair degree of immunity, the other two proved at autopsy to be carriers (liver and gall-bladder). The remaining 8 rabbits (638, 639, 646, 647, 648, 662, 663, 698) had served as controls for some other experiments and had been injected with various typhoidin preparations from 4 to 6 weeks previous to their use in Group 2. This treatment apparently had produced conditions in

the skin and blood serum of some of the rabbits which were not anticipated and which therefore made the interpretation of the results very difficult.

The preparation of the typhoidin used in this experiment was as follows: (1) *B. typhosus* Olsen* was inoculated into glycerin rabbit broth containing 2% Witte's peptone, and grown in shallow layers in several Blake bottles for 5 days. The contents of the various bottles were mixed, extracted at 60 C., concentrated, and precipitated as usual. A fowl typhoidin (Strain 605) was prepared in the same manner. These glycerin extracts were 3 days old when used.

(2) Typhoid and fowl-typhoid bacilli were similarly inoculated into Martin's peptone broth and grown in Blake bottles. Before concentration, sufficient pure glycerin was added to the mixture to make a 5% solution. A comparatively small amount of precipitate was obtained from these cultures, yet the antigenic value was about the same as for the other preparation described.†

As controls, we used sterile broth preparations treated in identical manner.

The results of this interesting test are shown in Tables 4a and 4b.

The preparations GI and GII produced, as will be noted, in most animals (except 662 and 663) very severe skin reactions, the degree of induration and diameter of the areola depending entirely on the amount of powder-suspension inoculated. Previous tests, or the amount or type of typhoidin applied intracutaneously, had no influence on the present degree of the reactions. The typhoidin was somewhat more intense than the fowl typhoidin. Unfortunately, only one rabbit (684) acted as a control and, judging from this single observation, the powder GI was slightly irritant to an unsensitized rabbit.

The infected rabbits (619 and 642) gave positive skin tests which persisted for 48 hours. In comparison with the large edematous areola of the sensitized rabbits and the immune rabbit (644), the reactions were small. In the light of some other experiments, to be discussed later, this result—for which we offer 2 possible explanations—was unexpected. Either the typhoidin preparation GI was detoxicated in some of the rabbits possessing a certain degree of immunity (Rabbits 644 and 663) or, what is perhaps more plausible, the anemic, emaciated, and infected animals (619, 642) for reasons thus far not investigated, responded poorly to cutaneous tests. We may recall here that similar observations have been made in tuberculinization of man and animal. Calmette, Breton, and Petit³¹ found a disappearance of the ophthalmic reaction with the beginning of cachexia in tuberculous rabbits.

* GI = typhoidin in 2% peptone broth = 20 e. u. per 0.001 gm. GII = fowl typhoidin in 2% peptone broth = 10 e. u. per 0.001 gm.

† FI = Martin's-broth typhoidin = 20 e. u. per 0.001 gm. FII = Martin's-broth fowl typhoidin = 33 e. u. per 0.001 gm.

³¹ Compt. rend. Soc. de biol., 1907, 63, p. 296.

TABLE 4a
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Animal	Infection and Result	Previous Test	Serologic Test, May 17, 1916		Hours
			Agglutination	Complement-Fixation	
619	<i>B. typhosus</i> H-125 carrier May 8, 1916		1:4000 —1:8000	0.003	24 48 72
638	Control	April 10, 1916 Typhoidin P.C. Paratyphoidin A Paratyphoidin B <i>B. coli</i>	1:100	0.05	24 48 72
639	Control	April 10, 1916 Typhoidin P.C. Paratyphoidin A Paratyphoidin B <i>B. coli</i>	1:40	0.1	24 48 72
644	<i>B. typhosus</i> H-125 April 13, 1916		1:1000 —1:2000	0.001	24 48 72
685	Control		1:20	0.1	24 48 72
698	Control	April 10, 1916 Typhoidin P.C. Paratyphoidin B Paratyphoidin A <i>B. coli</i>	1:40	0.05	24 48 72

The control powders, rich in peptones, were not absolutely inert; small indurated areolae developed with broth-control suspensions, becoming smaller, however, after 24 hours and even disappearing entirely in rabbits of poor reaction. Some rabbits seemed to respond more intensely than others, individual factors appearing to play a certain rôle. The possible criticism that the numerous skin tests on one and the same animal may influence one another, is not substantiated by our observations.

Preparations FI and FII, probably on account of the smaller peptone content, were less active in previously sensitized rabbits, yet not entirely inert on normal rabbits. We have already referred to the fact that, unfortunately, only one control rabbit was used in each lot of Group 2. It is not unlikely that Rabbit 685 was one of those susceptible animals which gave a very marked skin areola with edema following the injection of Typhoidin FI, a response which we have already described in Group-experiment 1. The presence of a marked

TABLE 4a—(Continued)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

DATE OF TEST: MAY 19, 1916

Typhoidin F		Control Powder	Fowl-Typhoidin G II		Control Powder
1:100	1:50		1:100	1:50	
(cm.) 1.2 x 1.2 1.3 x 1.3 N. P.	(cm.) 1.4 x 1.4 1.8 x 1.8 N. P.	(cm.) N. P. N. P. N. P.	(cm.) 0.9 x 0.9 1.1 x 1.1 N. P.	(cm.) 1.5 x 1.5 2.1 x 2.1 N. P.	(cm.) N. P. N. P. N. P.
2.7 x 2.7	3.8 x 3.8, raised 1.2	1 x 1	1.7 x 1.7	2.1 x 2.5	0.9 x 0.9
2.8 x 2.8	2.1 x 2.1, R. I.	0.5 x 0.5	0.9 x 0.9	2.4 x 2.5	0.6 x 0.6
N. P.	0.5 x 0.5, nodule	N. P.	N. P.	0.6 x 0.6	N. P.
2.4 x 2.4	2.9 x 2.9	1 x 1	1.4 x 1.4	1.5 x 1.5	0.9 x 0.9
1.8 x 1.8	2 x 2	0.5 x 0.5	0.5 x 0.5	0.8 x 0.8	0.8 x 0.8
0.7 x 0.7	1.3 x 1.3	0.5 x 0.5	N. P.	0.6 x 0.6	N. P.
2.5 x 2.5 2 x 2 N. P.	2.2 x 2.8 2.6 x 1.9 0.8 x 0.8, nodule	0.8 x 0.8 N. P. N. P.	1.5 x 1.5 0.7 x 0.7 N. P.	2.3 x 2.3 0.8 x 0.8 0.8 x 0.8, nodule	0.8 x 0.8 0.6 x 0.6 N. P.
Diffuse edema 1.6 x 3.1, edema N. P.	Diffuse edema 1.6 x 3.1 N. P.	N. P. N. P. N. P.	1 x 1 0.6 x 0.6 N. P.	1.1 x 1.1 1.4 x 1.4 N. P.	0.8 x 0.8 0.5 x 0.5 N. P.
3 x 3	3.4 x 3.4	0.9 x 0.9	1.6 x 1.6	2 x 2	1 x 1
2.5 x 2.5	2 x 2.5	0.6 x 0.6	1.6 x 1.6	2.2 x 2.2	0.7 x 0.7
1.4 x 1.4	1 x 2.5	N. P.	0.5 x 0.5	1.3 x 1.3	0.6 x 0.6

edema with slight hyperemia, induration, and increased heat is in striking contrast, however, with the typical cutaneous reactions in sensitized rabbits. Most of the rabbits reacted simultaneously to fowl typhoidin, and the criticism that some of the reactions were due to faulty intracutaneous technic and dosage of the suspensions is not well supported by the quantitative tests. The accuracy of our technic is shown by the fact that the areolae and the degrees of induration were dependent on the concentrations of the various powders. Small reactions with typhoidin, or their entire absence, were paralleled by a similar response with fowl typhoidin.

Analyzing Tables 4a and 4b more carefully, we note that previous injections of typhoidin produced in a certain percentage of animals a condition of the skin which resulted in the development of severe reactions subsequent to a new intracutaneous application of typhoidins rich in peptones and toxic split products. In our opinion, 2 factors are responsible for these conditions: in the first place it is possible that

TABLE 4b
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN
DATE OF TEST: MAY 19, 1916

Animal	Infection and Result	Previous Test	Serologic Test, May 17, 1916		Hours
			Agglutination	Complement-Fixation	
642	Control	April 13, 1916 <i>B. typhosus</i> 11 125 I. V., carrier	1:2000	>0.003	24 48 72
646	Control	April 17, 1916 Typhoidin 15 Fowl-typhoidin 4	1:40	0.05	24 48 72
647	Control	April 17, 1916 Typhoidin 15 Fowl-typhoidin 4	1:40	0.1	24 48 72
648	Control	April 17, 1916 Typhoidin 15 Fowl-typhoidin 4	1:100 —1:200	0.05	24 48 72
662	Control	April 25, 1916 Typhoidin E Paratyphoidin A Paratyphoidin B	1:40	0.05	24 48 72
663	Control	April 25, 1916 Typhoidin E Paratyphoidin A Paratyphoidin B	1:200 —1:400	>0.05	24 48 72
684	Control		1:20	0.1	24 48 72

a fair number of rabbits are capable of developing cutaneous hypersensitiveness to typhoidin and similar preparations—a response which is probably a phase of a general sensitization; or, secondly, on account of some individual factors or on account of the unsuitability of the preparations used, not every animal may react to repeated skin tests. Rabbits 662 and 663 illustrate the point that the typhoidin preparations used for sensitization may have some influence because, in contrast with Rabbits 646, 647, and 648, the reactions in these two rabbits were small. But it should be kept in mind that individual differences also play a part in the explanation of the intensities of the reactions noted in Rabbits 662 and 663.

The second factor, the hypersensibility of the rabbit skin to peptone and toxic split products also merits consideration. It is difficult to decide how regularly the condition occurs in animals until a large series of tests has been carried out. From experience with tuberculin

TABLE 4b.—(Continued)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN AND FOWL TYPHOIDIN

DATE OF TEST: MAY 19, 1916

Typhoidin G I		Control Powder	Fowl-Typhoidin F II		Control Powder
1:100	1:50		1:100	1:50	
(cm.) 1.8 x 1.8 0.9 x 0.9 Red spot	(cm.) 2 x 2 1.4 x 1.4 0.7 x 0.7	(cm.) N. P. 0.4 x 0.4 N. P.	(cm.) 1.5 x 1.5 0.9 x 0.9 N. P.	(cm.) 1.8 x 1.8 1.6 x 1.6 N. P.	(cm.) 0.7 x 0.7 0.6 x 0.6 N. P.
3.2 x 3.2 1.8 x 1.8 0.3 x 0.3	3.7 x 3.7, raised 0.5 2.3 x 2.9 0.8 x 0.8, indurated	0.9 x 0.9 N. P. Red spot	1.2 x 1.2 1.1 x 1.1 Red spot	1.8 x 1.8 1.6 x 1.6 Red spot	0.8 x 0.8 0.5 x 0.5 Red spot
2.9 x 2.9, edema; R. I. 1.5 x 1.5 0.3 x 0.3	3.6 x 3.6 1.6 x 1.6, indurated 0.5 x 0.5	1 x 1 0.8 x 0.8 Red spot	17. x 1.7 1.8 x 1.8 Red spot	1.4 x 1.4 1.9 x 1.9, I. R. 0.3 x 0.3	0.9 x 0.9 0.9 x 0.9 Red spot
2.4 x 2.4 1.4 x 1.4 0.5 x 0.5	3.5 x 3.5 1.5 x 2.7 1.1 x 1.1	0.8 x 0.8 0.6 x 0.6 N. P.	1.8 x 1.8 1.2 x 1.2 0.4 x 0.4	2 x 2 2 x 2 0.4 x 0.4	0.6 x 0.6 0.8 x 0.8 Red spot
0.5 x 0.5 0.3 x 0.3 0.2 x 0.2	3.6 x 3.6, edema 0.8 x 0.8 0.5 x 0.5, R. I.	0.6 x 0.6 0.8 x 0.8 0.3 x 0.3	1.3 x 1.3 N. P. N. P.	N. P. N. P. N. P.	0.6 x 0.6 N. P. N. P.
0.8 x 0.8 0.4 x 0.4 0.5 x 0.5	N. P. 0.4 x 0.4 Red spot	N. P. N. P. N. P.	0.6 x 0.6 1 x 1 0.3 x 0.3	N. P. 1.6 x 1.6 0.2 x 0.2	N. P. 0.3 x 0.3 N. P.
0.5 x 0.5 0.2 x 0.2 N. P.	0.6 x 0.6 0.4 x 0.4 N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.

we know that the rabbit is about three million times less sensitive to this preparation than is man. Preparations of bacterial extracts which give, in low dilutions, large nonspecific reactions in man are, as a rule, absolutely inert in the rabbit. Our observations in Experiment-group 2 would indicate that a hypersusceptibility of rabbits to peptone may occur, but in a limited percentage of animals only. When, however, preparations with the regular low peptone content are used, we have observed misleading reactions in rare instances.

From the experiments of Group 2 we may conclude that 5-day-old glycerin extracts of the typhoid and the fowl-typhoid bacillus containing 2% Witte's peptone or made from pig stomach and kept in shallow layers, contain a fair amount of antigenic substances. The intense reactions produced by them on immunized and previously 'typhoidinized' rabbits are, however, out of proportion to the antigenic

value of the preparation. These preparations also cause nonspecific edemas and reactions in normal rabbits and are therefore considered unsuitable for cutaneous tests.

It may be assumed that these preparations are irritant and non-specific primarily on account of the high endotoxin content which we know from previous experiments may develop in this type of medium and under the conditions of free exposure to oxygen, and as a result of the high concentration of alcohol-precipitable proteids. These substances—peptones, albumoses, and other protein split products—in all probability favor also the alcohol precipitation of the endotoxin by adsorption. As a rule, typhoid toxins or similar toxic protein split products are, according to von Stenitzer,³² not quantitatively precipitable by alcohol. In addition to these conditions, all these undesirable precipitable substances considerably dilute the antigenic substances demonstrable by fixation tests and probably are in part responsible for the low antigenic value of the extract (1 mg. of the typhoidin G and F).

The observations made in Group 2 suggested the use of glycerin extracts of young cultures in which the toxin-production is just commencing and which is, therefore, of low concentration. Furthermore, it was considered advisable to use different strains of *B. typhosus* for these cultures. Old stock cultures ordinarily grow very vigorously in glycerin veal broth, and the precipitate obtained is abundant in comparison with the poor growth of recently isolated strains. Also the toxogenic effect of various strains in one and the same medium is, in our opinion, not uniform. A low toxic strain with optimal growth would constitute the best organism for a suitable typhoidin. Pulay² suggested in his publication, the use of a 48-hour-old culture of a recently isolated strain. In his experience a 'typhin' prepared in this manner gave better and more distinct reactions than one made with an old strain.

In two series the value of these suggestions was tested. Extracts of other organisms were included to determine the sensitiveness of the immunized rabbits in general. In one series *B. coli* and *B. dysenteriae*, and in one, staphylococci and fowl-typhoid extracts, were used

EXPERIMENT-GROUP 3

Two lots, of 4 and 5 rabbits, respectively, together with 2 controls, were tested with various preparations on Feb. 23, 1916.

Lot I consisted of 4 rabbits (332, 337, 358, 360) which were immunized against

³² Handbuch d. Technik und Methodik d. Immunitätsforschung, 1908, 1, p. 200.

4 different strains of typhoid bacilli. Fifty and 109 days, respectively, following the last injections, the animals were in good health and, as could be judged by the serum reactions, still showed a fair degree of immunity.

The glycerin extracts used had the following antigenic value: Typhoidin 4 (Kuhn) 48 hours' growth (strain isolated in November, 1915), 10 e.u. per milligram.

Fowl-Typhoidin 4, (A. P. Smith) 5 days' growth, 50 e.u. per milligram. The 'staphylococcin' was prepared from a 48-hour-old culture of a recently isolated human strain of staphylococcus; its antigenic value was not determined. All three extracts used were prepared with a veal Witte's peptone glycerin (5%) broth. The results of the skin tests are shown in Table 5.

A glycerin extract prepared from a 48-hour-old culture of a recently isolated strain of *B. typhosus* with fair antigenic properties, caused, on intradermal injection of 1 mg., reactions which differed very little—if at all—from those produced in the controls. On the other hand, a fowl typhoidin prepared from a 5-day-old culture at the same time and with the same medium, caused the formation of areolae similar in size and intensity to those already described in the experiments of Group 1; the intensity of the reactions varied somewhat in different animals. The staphylococcus extract, which was absolutely inert as tested on several other control animals not mentioned in the protocol, caused in one rabbit (358) a well-defined, typical reaction which persisted for 48 hours. A small areola was also noticed in Rabbit 332, but as will be seen from Table 5, the reaction to fowl typhoidin in this animal remained for at least 48 hours, those to the staphylococcus extract having by that time nearly disappeared. An explanation of this nonspecificity, which is observed in a certain percentage of rabbits, will be attempted later when similar or identical observations with other extracts have been cited.

In Lot 2, 5 rabbits (335, 336, 355, 357, 361) had been immunized with varying amounts of typhoid antigens of different strains. Corresponding with the lapse of time (58, 102, and 107 days) after the last injection, the immunity was still well marked. One animal (357) suffered from a coccidiosis relapse and was emaciated and anemic on the day of the test; all the other animals were in good condition. Rabbits 335 and 336, which were older animals than 355 to 361, had been immunized with very small doses of very toxic, recently isolated strains.

Only extracts of 48-hour-old cultures in a medium of the same composition as that used on Lot 1, were used. The typhoidin Rawlings (Army strain) had an antigenic value of 20 e.u. per milligram. The dysentery strain, Shiga Do., was an old laboratory strain received from the Institut Pasteur, whereas the colon bacillus had been recently isolated from a case of cystitis. The results of the test are shown in Table 6.

TABLE 5

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN, STAPHYLOCOCCIN AND FOWL TYPHOIDIN
DATE OF TEST: FEB. 23, 1916

Animal	Immunization	Last Injection, 1915	Serologic Tests		Hours	Typhoidin 'Kuhn' (48)	
			Agglutination	Complement-Fixation		1:100	1:500
332	B. typhosus 46, 7 injections	Nov. 8	1:800	0.003	16	(cm.) 0.5 x 0.5	(cm.) 0.2 x 0.2
					24	N. P.	N. P.
					48	N. P.	N. P.
337	B. typhosus 48, 7 injections	Nov. 8		0.005	16	0.2 x 0.2	N. P.
					24	0.2 x 0.2	N. P.
					48	N. P.	N. P.
358	B. typhosus 79, 7 injections	Dec. 26	1:800	0.01	16	0.8 x 0.8	0.3 x 0.3
					24	0.6 x 0.6, R. I.	N. P.
					48	N. P.	N. P.
360	B. typhosus 90, 7 injections	Dec. 26	1:800	0.005	16	0.4 x 0.4	0.2 x 0.2
					24	0.5 x 0.5, S. I.	N. P.
					48	N. P.	N. P.
607	Control		1:40	0.1	16	0.3 x 0.3	N. P.
					24	N. P.	N. P.
					48	N. P.	N. P.

In Lot 2 the precipitate of a 48-hour-old typhoid culture of the Rawlings strain produced slight or no reactions at all in properly immunized animals. Only Rabbit 335 gave a reaction which could be considered positive. The striking absence of the least reaction in Rabbits 355, 357, and 361 is difficult to explain. The absence of reactions with other bacterial extracts suggests that either Rabbits, 355, 357, and 361 possessed no cutaneous hypersensitiveness, or that the test preparations were insufficiently active in the 1-mg. amounts injected. The first explanation is not very well supported by subsequent observations in which the same rabbits gave good and distinct reactions; it is apparent, however, that their sensitiveness must have been low, either on account of disease or because of the few injections, inasmuch as Rabbits 335 and 336, which reacted with typhoidin, gave very marked reactions also with the colon-bacillus and dysentery extracts. In comparison, the typhoidin areolae were small, but

TABLE 5.—(Continued)

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN, STAPHYLOCOCCIN AND FOWL TYPHOIDIN
DATE OF TEST: FEB. 23, 1916

Staphylo- coccin (48) 1:100	Fowl-Typhoidin 4 (5)		Control- Powder 4 1:10	Remarks
	1:100	1:500		
(cm.) 0.9 x 0.9, R. I. 0.3 x 0.3, R. I. N. P.	(cm.) 1.3 x 1.3, R. I. 1.2 x 1.2, W. D. 1.1 x 1.1, R. I.	(cm.) 0.2 x 0.2 N. P. N. P. N. P.	(cm.) N. P. N. P. N. P.	Re-injected March 2, 1916 Not a carrier
0.3 x 0.3 0.3 x 0.3, diffusely red N. P.	Diffuse red area 2 x 2 1.7 x 1.7, R. I.	1.9 x 1.9, R. I. 1.9 x 1.9, R. I. N. P.	0.3 x 0.3 0.2 x 0.2 N. P.	Re-injected March 2, 1916 Not a carrier
2.1 x 2.1, R. I. 1.9 x 1.9, R. I., W. D. N. P.	1.1 x 1.1 1.1 x 1.1 N. P.	0.2 x 0.2 N. P. N. P.	N. P. N. P. N. P.	Re-injected March 3, 1916 Carrier
0.3 x 0.3 N. P. N. P.	1.7 x 1.7, I. R. 2.2 x 2.2, W. D. 1.6 x 1.6, R. I.	Diffuse red area 0.9 x 0.9, I. R. N. P.	N. P. N. P. N. P.	Re-injected March 2, 1916 Carrier
N. P. N. P. N. P.	0.5 x 0.5 0.2 x 0.2 N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	

decidedly better than those produced by the preparation 'Kuhn' used on Lot 1. The absence of skin reactions in these three rabbits is probably the result of several factors combined. It is furthermore possible that the famous Rawling strain, which recently through the studies of Hooker³³ and Garbat³⁴ has been found to exhibit antigenic properties different from those of recently isolated typhoid strains, may also act abnormally in cutaneous tests. At present our data do not warrant such conclusions.

The positive reactions with extracts of *B. coli* and *B. dysenteriae* Do. in Rabbits 335 and 336 are interesting from many viewpoints. Both preparations were practically inert on normal animals. That these reactions were not the result of normal broth constituents is shown by the slight reactions (0.01 gm.) of broth precipitates produced

³³ Proc. Soc. Exper. Biol. and Med., 1916, 13, p. 139.³⁴ Jour. Immunol., 1916, 1, p. 391.

TABLE 6
RESULTS OF INTRACUTANEOUS TESTS IN GROUP 3, LOT 2
DATE OF TEST: FEB. 23, 1916

Animal	Immunization	Last Injection, 1915	Serologic Tests, Feb. 23, 1915		Hours	Typhoidin 'Rawlings' (48)	
			Agglutination	Complement-Fixation		1:100	1:500
335	B. typhosus 49, 7 injections	Nov. 8	1:600	0.02	16	(cm.) 0.8 x 0.8	(cm.) 0.4 x 0.4
					24	1.2 x 1.2, R. I.	0.9 x 0.9, S. I.
					48	0.5 x 0.5	0.3 x 0.3
336	B. typhosus 50, 7 injections	Nov. 8	1:2000 —4000	1.005	16	0.3 x 0.3	0.3 x 0.3
					24	0.5 x 0.5	0.2 x 0.2
					48	N. P.	N. P.
355	B. typhosus 73, 3 injections	Nov. 13	1:1000	0.01	16	N. P.	N. P.
					24	N. P.	N. P.
					48	N. P.	N. P.
357	B. typhosus 75, 6 injections	Dec. 27	1:2000	0.005	16	N. P.	N. P.
					24	N. P.	N. P.
					48	N. P.	N. P.
361	B. typhosus 94, 6 injections	Dec. 27	1:2000 —4000	0.005	16	N. P.	N. P.
					24	N. P.	N. P.
					48	N. P.	N. P.
608	Control		1:10	0.05	16	N. P.	N. P.
					24	0.2 x 0.5	N. P.
					48	N. P.	N. P.

on the skin of Rabbits 335 and 336. It is possible that these rabbits were very susceptible to toxic split products produced by *B. coli* and *B. dysenteriae* in the culture medium. Against this explanation is the absence of reactions in the control, Rabbit 608. In the light of experiments to be discussed in the next paper, these reactions may be explained as the result of nonspecific sensitization.

These group experiments do not very well support the contention of Pulay, that a 48-hour culture is just as suitable as a 5-day growth, and a recently isolated strain just as good as an old one. Furthermore, it is evident that the amount of extract units (e. u.) demonstrated by the complement-fixation test does not absolutely indicate the value of the preparation as an antigen.

The growth of the typhoid and staphylococcus strains in 48 hours was rather poor, and we suspect that the contents of the precipitate in antigenic bacterial proteins was correspondingly small. That the

TABLE 6.—(Continued)
RESULTS OF INTRACUTANEOUS TESTS IN GROUP 3, LOT 2
DATE OF TEST: FEB. 23, 1916

B. Dysenteriae Shiga (48) 1:100	B. Coli 4 (48)		Control- Powder 4 1:10	Remarks
	1:100	1:500		
(cm.) 2.2 x 2.2, R. I. 2.1 x 2.1, R. I. 1.8 x 1.8, S. I.	(cm.) 2.2 x 1.5, R. I. 1.8 x 1.8, I. R. 1.9 x 1.9, R. I.	(cm.) 0.9 x 0.9 1.1 x 1.1 N. P.	(cm.) 0.2 x 0.2 0.2 x 0.2 N. P.	Carrier
1.9 x 1.9, R. I. 2.5 x 2.5, R. I. 1.5 x 1.5, R. I.	2 x 2, D. I. 1.5 x 1.5, S. I. 0.6 x 0.6, R. I.	0.9 x 0.9 1.5 x 1.5 0.2 x 0.2	0.2 x 0.2 0.2 x 0.2 N. P.	Re-injected March 2 Carrier
N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	Re-injected March 3 Died intercurrent disease Not a carrier
N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	Died on February 25 Anemic and sick when tested Emaciated
N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	Re-injected March 2 Became carrier
N. P. 0.8 x 0.8 N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	

growth of the organism was slight is quantitatively evidenced by the weights of the precipitates.*

It is also quite possible that the insufficient growth of the micro-organisms did not produce those substances in the broth cultures which are essential for a distinct skin reaction. One point is quite certain from observations on Lots 1 and 2 of this group, and that is that a good fowl typhoidin will produce skin reactions in typhoid-immune rabbits, and that a few of these animals will also give reactions with extracts of *B. coli*, staphylococci, and *B. dysenteriae*.

SUMMARY

In this communication on cutaneous tests with glycerin extracts of broth cultures, we have endeavored to explain our methods of preparation of the antigenic powders. These studies have been made.

* Weight of dried precipitate of 10 c.c. of concentrated glycerin extract: *B. typhosus* (Kuhn), 48 hours' growth = 0.3990 gm. *B. coli*, 48 hours' growth = 0.4813 gm. *B. dysenteriae* Shiga, 48 hours' growth = 0.4442 gm. *Staphylococcus*, 48 hours' growth = 0.3890 gm. *B. typhosus*, No. 4 (Olsen), 5 days' growth = 0.6931 gm.

with a few exceptions, entirely with glycerin extracts of broth cultures. In the course of our observations it became clear that glycerin is not absolutely necessary for the preparation of good antigens, but we felt nevertheless that for comparative tests experimental data should first be collected with a preparation already known and analogous to the tuberculin and mallein, with which we have had considerable experience.

In this study we have attempted also to obtain some information regarding the composition and reaction of the medium, the growth of the bacteria and their toxic split products, the age of the culture, the type of strain, and other factors with reference to the influence they may have on the suitability of the resulting product for these tests.

It has been found, for instance, that a high peptone content together with rich exposure of the broth to oxygen, will produce typhoidin powders which are very irritant and active for nonimmune or sensitized rabbits. Young 48-hour-old cultures of recently isolated strains, on the other hand, produce powders which are inactive in dilutions ordinarily suitable for old (5- to 15-day) cultures. Vigorously growing old laboratory strains of the typhoid bacillus and other bacteria give, at times, active antigenic powders.

With the desire to reduce the production of toxic split products and to increase the antigenic substances, we used completely filled flasks containing potato broth. When the bacteria were grown in this manner, the protein-sparing action of the carbohydrates and the long incubation of the cultures resulted in extracts which were practically atoxic as tested by intravenous inoculation of sterile filtrates (in several tests only amounts of from 8 to 10 c.c. produced typical death). The studies of Theobald Smith and Ten Broeck³⁵ on the fowl-typhoid toxin, those of Kraus and Stenitzer³⁶ on the typhoid toxin, and our findings with the B.-enteritidis and B.-paratyphoid-A toxins, suggested the use of cultures over 10 days old, because, as a rule, the maximal amount of toxin is produced between the 8th and the 10th day.

Experience has shown that fractional extractions at a temperature increasing from 40 to 60 degrees, resulted, just as in the case of tuberculin, in preparations which were more active than those concentrated rapidly at high temperatures. Filtration and rapid treatment with alcohol, favor the production of salt-solution-soluble powders. Unfiltered extracts, or those washed repeatedly with alcohol, are fre-

³⁵ Jour. Med. Research, 1914-1915, 31, p. 523.

³⁶ Ztschr. f. Immunitätsf., 1909, 3, p. 646.

quently insoluble. In form of suspensions such preparations act non-specifically as irritants.

We recommend the titration of the glycerin extracts by means of the complement-fixation test, primarily to determine the antigenic value and, secondarily, to follow the deterioration of a preparation. We have not as yet made full use of this method, but we record a few suggestive observations. The value of typhoidin or broth-culture extract should also be tested as to its sensitizing value by inoculations into rabbits in a dosage of 0.1 to 0.01 gm. It is of interest to bear in mind, however, that powders perfect in every respect may prove on animal inoculation to possess low antigenic properties.

In quantities of 0.001 to 0.0002 gm. a perfect preparation was found to be inert on the skin of normal untreated rabbits. In immunized, infected, or otherwise sensitized animals, typical inflammatory areolae—characterized by hyperemia, induration, and increased warmth—developed at the site of injection. Central papules with yellowish centers developed only with very concentrated and insoluble powders, and are in our experience the result of pyogenic action of the concentrated bacterial proteins. It is self-explanatory that such preparations are unsuitable for careful skin tests. In our experiments we successfully used various dilutions of one antigenic powder. These quantitative tests not only control the technic of injection, but they also yield, in many instances, information concerning the gradual diminution in the intensities of the reactions, thus making decisions possible in regard to specificity.

The use of control powders in fairly high concentration—which in reality demonstrate only the individual susceptibility of the rabbit skin to peptone and broth elements—was amplified by control powders which were made with various other bacterial cultures, as *B. coli*, *B. dysenteriae*, etc. The reactions which resulted from the use of such powders indicated more precisely the status and nature of the cutaneous hypersensitiveness of a rabbit to bacterial protein. Subsequent papers will deal more extensively with these subjects.

CONCLUSIONS

Glycerin broth-culture extracts of the typhoid bacillus precipitated with alcohol can be used for cutaneous tests on rabbits, provided the culture is poor in peptones and the organism is grown for a long time with a limited exposure to oxygen.

The proper amount of bacterial extract powder to produce distinct reactions in rabbits varies between 0.0001 and 0.0002 gm. The reaction apparently depends on other antigenic substances than those which can be titrated by means of the complement-fixation test.

Powders prepared of the sterile broth serve as controls only for the hypersensitiveness of the rabbit skin to broth precipitates and extractives. Broth cultures of other bacteria are recommended as additional controls.

THE INTRACUTANEOUS 'TYPHOIDIN' REACTION*

II. THE NATURE AND SPECIFICITY OF THE REACTION

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In the previous communication we made observations on positive skin reactions in typhoid-immune rabbits by using, as controls, glycerin broth extracts of bacteria other than typhoid. In the course of these studies we obtained results which suggested further experiments which might throw some additional light on the nature, and possibly also on the mechanism, of cutaneous hypersensitiveness and allergic skin reactions in general.

Several theories have been advanced to explain cutaneous hypersensitiveness to tuberculin, and, without additional experimental evidence, workers have used the results of studies made on other skin reactions to explain the phenomena which pertain to the skin tests which we have under consideration.

It has always been a debatable question whether the allergic tuberculin reactions are analogous to the anaphylactic reaction with serum, and whether similar skin reactions are also of the same nature. Doerr,¹ in his summary on allergy and anaphylaxis, considers the question an open one as to whether or not all the local reactions to extracts or antigenic preparations should be considered as a local anaphylaxis of the reacting individual or animal, to the protein of the disease-producing agent.

From the facts presented by numerous workers, it is certainly not unlikely that hypersensitiveness in the form of the local tuberculin reactions to various forms of antigen derived from the tubercle bacillus is, in reality, an anaphylactic phenomenon. Observations of von Pirquet² and others on local acquired hypersensitiveness indicate that this is more analogous to the phenomenon of sensitization than to that of hypersusceptibility to toxin. Furthermore, to support this theory a few successful attempts have been made at a passive transfer of hyper-

* Received for publication December 4, 1916.

¹ Handb. d. pathogen. Mikroorganismen, 1913, 2, p. 1102.

² Handb. d. Technik u. Methodik d. Immunitätsf., 1908, 1, p. 1050.

sensitiveness to tuberculin. However, the opponents of this conception of the nature of the tuberculin reaction have presented evidence that the active principle of tuberculin is not a specific bacterial protein, but a product closely allied to the true toxins, and that tuberculous guinea-pigs do not react as regularly to pure tuberculoprotein as to tuberculin.

From the literature on hand one may say that the experimental data are, in many respects, in accord with the theory that the tuberculin reaction is the result of sensitization. For all other forms of local allergic reaction, to mallein, 'sporotrichosine,' 'luetin,' and 'typhoidin,' the evidence thus far collected is incomplete and does not as yet permit of conclusions. In some instances, as for example in the luetin reaction, the mechanism of the skin efflorescence is, in the light of recent observations, open to various interpretations. The report of Sherrick³ that persons treated with iodine will react with luetin, is, to be sure, of extreme interest in this connection.

Gay and Claypole,⁴ in discussing the mechanism of local reactions, state that their experiments indicate that the typhoidin reaction is due to an interaction of antigen and antibody. They do not speak of a phenomenon of sensitization, but consider the positive typhoidin reaction to bear a distinct relation to protection against typhoid fever. Nichols,⁵ however, regards the skin reaction as an anaphylactic phenomenon due to sensitization to typhoid protein, which is lasting and less specific than true immunity. Similarly definite are the conclusions of Kolmer⁶ in recent studies on various skin tests. He speaks of cutaneous anaphylaxis to typhoidin, and luetin, and hypersensitiveness to diphtheria protein, without presenting evidence that all these reactions are the result of a mechanism similar to that of anaphylaxis. We feel that it is not quite appropriate to regard all of his reactions as manifestations of anaphylaxis, more particularly in the case of his experiments with a "broth-culture typhoidin," in which he obtained a "high percentage of nonspecific reactions."

These experiments, however, support our suggestions that all the typhoidin tests should be repeated with pure bacterial proteins if that were possible or—as long as we are unable to isolate these—with the various fractions.

³ Jour. Am. Med. Assn., 1915, 65, p. 404.

⁴ Arch. Int. Med., 1914, 14, p. 699.

⁵ Jour. Exper. Med., 1915, 22, p. 780.

⁶ Jour. Immunol., 1916, 1, pp. 409, 429, 501, and 571.

Thus far only one form of antigen derived from the typhoid bacillus has been employed for the skin test on man; namely, the complex broth-culture precipitate. It must be demonstrated by further investigations whether this form of antigen is equivalent to the typho-protein. Since in our fundamental work on typhoidin with glycerin or plain broth-culture extracts or precipitates, our results proved to be very promising (with one exception), we considered it advisable to continue their use in our next series of experiments. Also, inasmuch as nonspecific reactions described in the 1st paper, were all obtained with extracts, it was imperative that subsequent control experiments should be conducted with an antigen made in similar way.

In our pursuit, experimentally, of these nonspecific reactions, we found evidence that the typhoidin reactions are the result of sensitization, and therefore to be explained by the theories applied to anaphylactic reactions in general.

Following the observations made in connection with Groups 1 and 3, as reported in our 1st paper, we planned several experiments in which rabbits protected against various kinds of microorganisms were to be tested with typhoidin and similar extracts. For these tests the animals of Group 1 were chosen.

EXPERIMENT-GROUP 1

Two series of tests were conducted with different typhoidin preparations.

Series 1.—Eight healthy rabbits with varying degrees of immunity against *B. bipolaris-septicus*, Morgan's bacillus, *B. dysenteriae* Flexner 12, were tested on Feb. 23, 1916. From 2 to 6 months had elapsed since these animals had received their last inoculation. Some had received, over a period of 3 months, as many as 11 injections of heat-killed or living organisms. The details of immunization and of the concentration of the immune bodies at the time of the test, are given in Table 1.

The glycerin extract, Typhoidin 3 (Olsen), contained 33.3 extract units (e.u.) per 0.001 gm., whereas Fowl-typhoidin 3, tho over 1 month old, had an antigenic value of 50 e.u. per 0.001 gm.

No determinations of antigenic value were possible in the case of the extracts of staphylococci and of *B. paratyphosus* B (homo). The first preparation was obtained from a 48-hour-old culture. The paratyphoid extract was used in quantities of 0.001 and 0.0002 gm.

The results of the experiment with Series 1 are presented in Table 1. Rabbits 462, 487, 489, 545, 561, 562, 567, not immunized against the typhoid bacillus, developed in 48 hours a skin reaction to typhoidin, which was in every respect typical. The areolae, which were

TABLE 1
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN PREPARATIONS (SERIES 1)
DATE OF TESTS: FEB. 23, 1916

Rabbit	Immunization	Last Injection	Serologic Tests		Hours	Typhoid-in 3 Olsen 1:100	Control-Powder 3 1:10
			Agglutination	Complement-Fixation			
462	Hemorrhagic septi- cemia. 6 injections of cultures and exudates	Aug. 27, 1915		0.003	16	(em.) 1.8 x 1.8, R. I.	(em.) 0.3 x 0.3
					24	1.9 x 1.9, W. D.	0.2 x 0.2
					48	1.9 x 1.9, R. I.	N. P.
487	B. abortus-equinarius 9. 8 injections	Jan. 3, 1916	1:2000	0.005	16	1.5 x 1.5, R. I.	0.4 x 0.4
					24	2.5 x 2.5, R. I.	0.2 x 0.2
					48	1.5 x 1.5, R. I.	N. P.
489	B. dysenteriae Flexner 12. 11 injections of living organisms	Dec. 16, 1915	1:100	0.05	16	2.8 x 2.8, R. I.	0.2 x 0.2
					24	2.7 x 2.7, S. I.	N. P.
					48	2.2 x 2.2, I. R.	N. P.
505	B. suispestifer Voldagsen. 11 injections	Dec. 16, 1915		0.001	16	0.2 x 0.2	N. P.
					24	0.2 x 0.2	N. P.
					48	N. P.	N. P.
545	Morgan's bacillus 1. 8 injections	Nov. 4, 1915	1:800	0.005	16	1.5 x 1.5, R. I.	0.3 x 0.3
					24	1.5 x 1.5, R. I.	0.4 x 0.4
					48	1.1 x 1.1, R. I.	N. P.
561	B. dysenteriae Hiss-Y-Russell. 6 injections	Dec. 16, 1915	1:600	0.05	16	0.5 x 0.5, R. I.	0.2 x 0.2
					24	0.8 x 0.8, R. I.	0.2 x 0.2
					48	1.8 x 1.8, R. I.	N. P.
562	Fowl typhoid D 4, 7 injections of living organisms	Dec. 16, 1915	1:200	0.01	16	1.6 x 1.6, R. I.	0.3 x 0.4
					24	1.5 x 1.5, R. I.	0.2 x 0.2
					48	1.4 x 1.4, R. I.	N. P.
567	Fowl typhoid Smith. 6 injections	Dec. 16, 1915	1:600	0.001	16	2 x 2, D. O.	0.2 x 0.2
					24	2.8 x 2.8, R. I.	0.4 x 0.4
					48	2.1 x 2.1, R. I.	N. P.
609	Control		1:20	0.05	16	0.2 x 0.2	N. P.
					24	0.2 x 0.2	N. P.
					48	N. P.	N. P.

KEY TO ALL TABLES

W. D. = well-defined areola
R. I. = deep purplish, indurated area
I. R. = indurated red area
S. I. = very slight induration
D. O. = diffuse edema

TABLE 1—Continued
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN PREPARATIONS (SERIES 1)
DATE OF TESTS: FEB. 23, 1916

Control-Powder 4 1:10	Staphylo- cocci 4 1:100	Paratyphoidin B (homo) 3		Fowl-Ty- phoidin 3 1:100	Remarks
		1:100	1:500		
(cm.) 0.2 x 0.2	(cm.) 0.4 x 0.4	(cm.) 1.9 x 1.9, R. I.	(cm.) 1.5 x 1.5, Sl. indur.	(cm.) 1.7 x 1.7, R. I.	Carrier on injection of B. typhosus
0.2 x 0.2	0.2 x 0.2	2.5 x 2.5, R. I.	1.4 x 1.4, R. I.	1.8 x 1.8, R. I.	
N. P.	N. P.	2.5 x 2.5, R. I.	0.9 x 0.9	1.4 x 1.4, R. I.	
0.2 x 0.2	0.2 x 0.2	0.4 x 0.4	0.4 x 0.4	2.6 x 2.6, S. I.	
N. P.	0.2 x 0.2	0.4 x 0.4	0.4 x 0.4	2.8 x 2.8, S. I.	
N. P.	N. P.	N. P.	N. P.	1.8 x 1.8, S. I.	
0.2 x 0.2	1.2 x 1.2, D. O.	0.4 x 0.4	0.2 x 0.2	0.5 x 0.5	Died from intoxication on re-injection
N. P.	1 x 1	0.2 x 0.2	0.2 x 0.2	0.2 x 0.2	
N. P.	0.5 x 0.5, R. I.	N. P.	N. P.	N. P.	
0.4 x 0.4	N. P.	0.4 x 0.4	0.4 x 0.4	0.4 x 0.4, S. I.	
N. P.	N. P.	0.2 x 0.2	0.2 x 0.2	0.2 x 0.2, S. I.	
N. P.	N. P.	N. P.	N. P.	0.2 x 0.2	
0.4 x 0.4	1.8 x 1.8, W. D.	0.9 x 0.9	0.6 x 0.6, R. I.	1.7 x 1.7, S. I.	Re-injected March 2. Carrier
0.6 x 0.6	2.2 x 2.2, indurated	1.4 x 1.4, R. I.	1.1 x 1.1	1.6 x 1.6, R. I.	
N. P.	1.7 x 1.7, R. I.	0.5 x 0.5, R. I.	0.2 x 0.2, R. I.	0.5 x 0.5	
0.2 x 0.2	0.3 x 0.3	N. P.	N. P.	N. P.	
N. P.	0.2 x 0.2	N. P.	N. P.	N. P.	
N. P.	0.2 x 0.2	N. P.	N. P.	N. P.	
0.2 x 0.2	1 x 1, D. O.	1.5 x 1.5, R. I.	0.2 x 0.2	1.8 x 1.8, R. I.	Re-injected March 3. Not a carrier
N. P.	0.6 x 0.6, red area	1.8 x 1.8, R. I.	N. P.	1 x 1, R. I.	
N. P.	0.2 x 0.2	1.2 x 1.2, R. I.	N. P.	0.6 x 0.6, R. I.	
0.3 x 0.2	0.3 x 0.3	2 x 2, D. R.	0.5 x 0.5	0.6 x 0.6	
N. P.	0.5 x 0.5	1.5 x 1.5, S. I.	0.8 x 0.8, D. R. area	1 x 1	
N. P.	0.2 x 0.2	0.8 x 0.8	0.3 x 0.3	1.6 x 1.6, W. D.	
0.2 x 0.2	0.2 x 0.3	0.4 x 0.4	N. P.	N. P.	Died from intercurrent dis- ease following injection with B. typhosus Not a carrier
N. P.	0.3 x 0.3	0.2 x 0.2	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	N. P.	

KEY TO ALL TABLES—Continued
S. O. = slight edema
D. R. I. = diffusely indurated and red
D. R. area or D. R. A. = diffuse red blush
S. spot = small spot
N. P. = Needle puncture, traumatic reaction

well-defined and indurated, persisted over 48 hours. This very active preparation possessed little primary toxicity, as is shown by the reaction on Control 609 (compare also Rabbit 627, Paper III, Experiment-group 1). Rabbits 562 and 567, being immunized against fowl-typhoid organisms, and 487, against a paratyphoid-like organism, may be considered as sensitized to the bacterioprotein of organisms closely related to the typhoid bacillus, and from the observations in previous experiments, it was expected that they might respond to typhoidin in a way comparable with group reactions in serologic tests. That Rabbit 462, immunized* against a microorganism not in the least immunologically related to the typhoid bacillus should respond to typhoidin, is at first difficult to understand. The same can be said with regard to Rabbits 489, 545, and 561, which had been immunized with paradysentery or allied organisms. These reactions are, in our opinion, the expression of a hypersensitiveness of the rabbit skin to bacterial proteins or toxins, induced by extensive immunization with living organisms. This condition is nonspecific, and not explained by so-called group reactions, because Rabbits 489 and 545 reacted also to staphylococcus extracts, and Rabbits 462 and 545 to paratyphoid-B extracts. The striking gradation in the degree of reaction in these two animals to paratyphoid-B extracts, shows again how valuable quantitative tests may be in deciding whether a reaction is accidental or actually due to the powder injected.

It is quite evident that animals thoroughly immunized with bacteria not the least related immunologically, will respond to various intracutaneous glycerin extracts. The degree of the reaction depends—as already shown in Experiment-group 3 of our 1st paper—on the type of the preparations. Extracts of 48-hour-old cultures are less active and, as a rule, they give irregular results. The fowl-typhoidin preparation, being the extract of a 5-day-old culture, gave reactions in 5 of 8 animals, which reacted also to typhoidin. The reactions were less intensive, however, because the preparation was not made in shallow layers as was the typhoidin. Only in Rabbit 567, immunized against fowl-typhoid organisms, did the reaction persist for 48 hours; in the other control animal, 562, the areola disappeared after the first 24 hours.

Three animals (489, 545, and 562) reacted with a weak staphylococcus extract; in 545 the reaction was in accord with the general

* The serum of this animal protected mice and rabbits against an infection with large doses of peritoneal exudate containing *B. bipolaris-septicus*.

hypersensitiveness to other bacterial proteins. In Rabbits 489 and 562 the reactions were so slight and transitory as to be doubtful, and possibly were due to faulty technic or traumatism.

Series 2.—Nine healthy rabbits, possessing a fair degree of immunity against *B. typhosus* (320, 322), fowl-typhoid organisms (404, 426), *B. pullorum* (410), *B. abortus-equi* (479), yeasts (424, 510), and organ extracts (560), respectively, were tested with various glycerin extracts. From 129 to 250 days had elapsed since these rabbits had received their last injections of antigen. Most of the animals were immunized with more than 8 injections, some receiving as many as 19. The details of the condition of immunity and of the procedure of immunization are given in Table 2.

Two rabbits (597 and 604) were infected with tuberculosis and sporotrichosis, respectively, subsequent postmortem examination demonstrating that the infection had taken place. Only 2 rabbits (662 and 663) were used as controls.

The typhoidin preparations E were used in the tests. Preparation E-I consisted of a 25-day-old glycerin potato broth, extracted and precipitated as usual; this preparation had an antigenic value of 20 e.u. per 0.001 gm. of powder. One portion of the broth culture was heated at 60 C. and autolyzed at 45 C. for 4½ hours, then filtered through hardened paper, preserved with 0.5% carbolic acid, and used undiluted as Antigen E-II.

A staphylococcus extract was prepared in the same manner as E-I. The two paratyphoid A and B powders, marked P.C., had been similarly prepared, but were about 1 month old when used. They proved to be specific and fully potent when subsequently tested.

The results of this series of tests are shown in Table 2. The typhoid-immune rabbits (320 and 322) gave fair skin reactions with both typhoidin preparations; the preparation E-II gave somewhat more intense areolae, and the induration persisted in Rabbit 322 for at least 48 hours. The same rabbit also responded more intensively to the paratyphoid-A and -B extracts than did Rabbit 320. Both rabbits had been immunized by the same process, and serum immune bodies were present in about equal concentration.

The rabbits immunized with bacterial antigens other than typhoid, all gave reactions with typhoidin which could not be distinguished from the skin reactions of Rabbits 320 and 322. However, Preparation E-I produced again somewhat less intense reactions. In 3 hyper-immune rabbits (404, 410, and 424) no dermatic response to typhoidin could be recorded. Of these three rabbits, 410 reacted to the paratyphoid-B extract, 424 to the staphylococcus extract and both typhoidin preparations, while 404 reacted to neither. In some of the rabbits, for example, 510 and 479, the diameter of the areola was greater and its induration more intense than in the typhoid-immune rabbits, and yet both these animals were immunized with organisms which botani-

TABLE 2
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN PREPARATIONS (SERIES 2)
DATE OF TESTS: APRIL 25, 1916

Rabbits	Immunization or Infection	Time Interval Since Last Injection	Serologic Tests		Hours
			Agglutination	Complement-Fixation	
320	Infection with <i>B. typhosus</i> 3. 7 injections	Nov. 8, 1915 153 days	1:4000	0.005	24 48
322	<i>B. typhosus</i> 15. 7 injections	Nov. 8, 1915 153 days	1:1000 —2000	0.005	24 48
404	<i>Bacillus of fowl-typhoid</i> Smith. Since March, 1915, 19 injections	Sept. 22, 1915 192 days	1:80	<0.02	24 48
410	<i>B. pullorum</i> III, 17 injections	Sept. 22, 1915 192 days	1:80	0.02	24 48
424	Yeast I. V. 14 injections	Sept. 14, 1915 201 days	1:20 (Olsen)		24 48
426	<i>Bacillus of fowl-typhoid</i> -A. P. Since April, 1915, 14 injections	Sept. 22, 1915 192 days	1:100	<0.02	24 48
479	<i>B. abortus</i> equi. 8 2 injections of toxin	Aug. 12, 1915 250 days	1:60	0.05	24 48
510	Yeast Curtiss. 5 large doses	Sept. 14, 1915 250 days	1:40 (Olsen)	0.01 (50%)	24 48
560	Submaxillary gland of dog	Dec. 16, 1915 129 days	1:40	0.05 (50%)	24 48
597	Tuberculosis	Feb. 17, 1916 Infected	(1:400?)	0.02 (50%)	24 48
604	Sporotrichosis	Feb. 17, 1916 Infected	1:40	0.1	24 48
662	Control		1:40	0.05 (50%)	24 48
663	Control		1:5	0.05 (50%)	24 48

TABLE 2—Continued
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN PREPARATIONS (SERIES 2)
DATE OF TESTS: APRIL 25, 1916

Typhoidin E		Control-Powder E 1:50	Staphylo- cocci E 1:100	Paraty- phoidin A, P. C. 1:100	Paraty- phoidin B, P. C. 1:100	Remarks
1:100	Broth Undiluted					
(cm.) 1.3 x 1.3 0.8 x 0.8	(cm.) 1.8 x 1.8 1.3 x 1.3	(cm.) 0.8 x 0.8 Red spot	(cm.) Red spot 0.6 x 0.6	(cm.) 1.3 x 1.3 Red spot	(cm.) 1.5 x 1.5 1.5 x 1.5	Re-injected on May 3. Car- rier
1.8 x 1.8 1.8 x 1.8	2.3 x 1.6 1.8 x 2.4	Red spot Red spot	Red spot 0.8 x 0.8	1 x 1 0.8 x 0.8	1.8 x 1.8 1.8 x 1.8	Re-injected on May 3. Not a carrier
N. P.	1.8 x 1.8	N. P.	0.9 x 0.9	N. P.	N. P.	Re-injected on May 3. Not a carrier
N. P.	0.8 x 0.8	N. P.	Red spot	N. P.	N. P.	
Red blush Red blush	1 x 1 1.3 x 1.3	N. P. N. P.	N. P. N. P.	N. P. N. P.	1.6 x 1.6 2.1 x 2.1	Re-injected on May 3. Died from infec- tion May 12
N. P.	D. R. A.	N. P.	1 x 1	1.7 x 1.7	1.3 x 1.3, yellow center	
N. P.	1.8 x 1.8	N. P.	0.5 x 0.5	1.7 x 1.7	0.5 x 0.5	
1.5 x 1.5	1.4 x 1.4	N. P.	0.8 x 0.8	0.8 x 0.8	Red area	Re-injected on May 3. Not a carrier
1.9 x 1.9	1.5 x 1.5	N. P.	1 x 1	0.8 x 0.8	1.8 x 1.8	
Red area 1.9 x 1.9, indurated white area	Indurated red area 2.1 x 2.1	N. P. N. P.	N. P. N. P.	N. P. N. P.	N. P. N. P.	
Red area 2.4 x 1.5, R. I.	Red area 2.1 x 2.1, R. I.	N. P. N. P.	N. P. N. P.	N. P. N. P.	N. P. N. P.	
N. P.	2 x 2, nodule, indurated	N. P.	N. P.	N. P.	N. P.	
N. P.	Red spot	N. P.	N. P.	N. P.	N. P.	
1.3 x 1.3, R. I.	1.5 x 1.5	Red area	N. P.	1.2 x 1.2	1 x 1	
1.5 x 1.5, R. I.	1.9 x 1.9	0.6 x 0.6	0.5 x 0.5	1.4 x 1.4	1 x 1	
1.5 x 2 1.7 x 1.7	1.8 x 1.3 1 x 1.5	N. P. N. P.	1.3 x 1.3 0.4 x 0.4	2.3 x 1.5 1.4 x 2	1.5 x 1.5 1.7 x 1.7	
1.5 x 1.5, areola puffy	N. P.	N. P.	N. P.	N. P.	N. P.	
0.6 x 0.6, red area	Red area	N. P.	N. P.	N. P.	N. P.	
N. P.	N. P.	N. P.	N. P.	Areola 0.5 x 0.5	N. P.	
N. P.	N. P.	N. P.	N. P.	N. P.	N. P.	

cally are not related to *B. typhosus*. It is possible that these skin reactions were due to toxic substances in the broth, for one control, 662, gave a slight reaction in the first 24 hours with E-I. In the immunized rabbits, however, well-marked and well-defined reactions were present after 24 hours, at a time when, in our experience, the toxogenic effect of the typhoidin powders would, as a rule, have disappeared. One might offer another and possibly better supported explanation for these reactions in that these rabbits became generally sensitized to some bacterial or vegetable (yeast) protein common to all organisms, and that the skin phenomena were the result of an anaphylactic reaction. For most of the hyperimmune rabbits this probably holds true, but for Rabbit 479 (Strain 8) this is, at first glance, unlikely, because the amount of bacterial protein inoculated was very small, and, as we became convinced from other experiments, was not sufficient to sensitize guinea-pigs. We found later, however, that even very small amounts of typhoproteins introduced intradermally for tests in normal healthy rabbits, are capable of producing a condition which gives rise to typical skin reactions on subsequent application of typhoidin. Rabbit 479 had been inoculated subcutaneously with a toxic broth filtrate of an organism closely allied to the paratyphoid-A bacillus, and in this case a group reaction with the typhoid antigen was therefore also possible.

In Experiment-group 1, Series 2, it is not apparent how much the degree of immunity and the time interval between the last injection and the skin test influenced the intensity of the reaction. From other experiments on rabbits, it is evident, however, that cutaneous hypersensitiveness if once acquired is never entirely lost, but will show frequent fluctuations in intensity, which are probably the result of factors unknown. A certain percentage of rabbits, just as has been observed in man, will never develop a hypersensitiveness even when injected at regular intervals with large amounts of bacterial substances. It is not unlikely that Rabbit 404 exemplifies this point, but it is also possible that the refractory condition of this animal was the outcome of over-immunization, which may result, as is generally known, in complete loss of power to produce antibodies.

Rabbits 597 and 604, infected with tuberculosis and sporotrichosis, respectively, gave well-defined skin reactions. It is interesting to note that the extensively tubercular rabbit, 597, also possessed agglutinins

for *B. typhosus*. These observations support the findings of Kraus⁷ and others in regard to tubercular human beings.

Cutaneous hypersensitiveness of tubercular persons to tuberculin and other bacterial extracts has always been one of the strong features which supported the conception of the tuberculin reaction as non-specific. We have made further inquiries into the nature of these reactions on other animals, and have collected further interesting data which will be considered later, in the discussion of these experiments. The presence of agglutinins cannot be the outcome of a mixed infection, as in man,⁸ but is probably the result of a nonspecific response of the antibody-forming organs, and represents a condition of antibody-production to which our attention has been recently directed by non-specific intravenous treatment with colloidal substances (Jobling⁹).

The animals of this group demonstrate the hitherto unknown fact that a certain percentage of rabbits, highly immunized with various living and dead bacterial suspensions, will give skin reactions following the intradermal introduction of glycerin broth-culture extracts of microorganisms which immunologically are in no way related to the bacteria used in the immunization. These nonspecific reactions persist, frequently, up to the 48th hour. They may be the result of cutaneous hypersensitiveness to certain elements of the bacterial protein. Some are due, perhaps, to individual hypersusceptibility to toxic elements of the glycerin extract. These conditions of the skin have thus far been noted in immunized and infected rabbits only.

EXPERIMENT-GROUP 2

The observations made in Group 1 have been verified in another series of tests and enhanced by the use of different types of preparations. In the light of the explanation which Stokes¹⁰ offered for the luetin reactions, it was considered of interest to test also a colloidal substance, like agar, in a parallel series with the regular extract powders. Group 2 represents one of the experiments prepared for this purpose.

Twelve healthy rabbits previously immunized with various bacterial suspensions and vaccines, and 3 control animals (646, 647, 648) were used in the test. Rabbits 328, 329, and 300, which had been intensely vaccinated against the

⁷ München. med. Wehnschr., 1910, 57, p. 437.

⁸ See the publications of Roth, *Centralbl. f. Inn. Med.*, 1910, 21, p. 1, and Kreuker, *München. med. Wehnschr.*, 1909, 56, p. 1017.

⁹ *Jour. Am. Med. Assn.*, 1916, 66, p. 1753.

¹⁰ *Jour. Infect. Dis.*, 1916, 18, p. 415.

TABLE 3
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN PREPARATIONS (GROUP 2)
DATE OF TESTS: APRIL 17, 1916

Rabbit	Immunization	Date of Last Injection	Serologic Tests		Hours
			Agglutination	Complement-Fixation	
313	Dog serum (27.5 c.c.) 4 injections	Jan. 25, 1916	0	0.1 (50% with B. typhosus)	24
					48
316	Dog serum (27.5 c.c. I. V.) 4 injections	Jan. 25, 1916	1:20 (Olsen)	0.1 (50% with B. typhosus)	24
					48
328	B. typhosus 20 (toxic strain) 6 injections	Nov. 8, 1915	4/17 1:1000	0.02	24
					48
329	B. typhosus 32 (toxic strain) 6 injections	Nov. 8, 1915	4/17 1:1000	0.02	15
					24
330	B. typhosus 38 7 injections	Nov. 8, 1915	4/17 1:1000-2000	0.01	48
					15
447	B. bipolaris-septicus 97 6 injections of living organisms and exudate	Sept. 28, 1915	4/17 0 (bipolar 49) 1:20 (B. ty- phosus)	0.01 (with Iowa I)	24
					48
463	Hemorrhagic-septi- cemic vaccine 7 injections	Sept. 28, 1915	4/17 1:200 (Iowa I) 1:200 (B. ty- phosus 49)	0.1 (50% with B. typhosus) 0.003 (with B. bipolaris)	24
					48
484	Yeast (sputum) 6 injections	Sept. 14, 1915	4/17 0	0.05 (50% with B. typhosus)	24
					48
492	B. pullorum (Berkeley) 13 injections	Dec. 3, 1915	4/17 1:60	0.05 (B. pullorum)	24
					48
495	B. avisepticus 5 injections	Sept. 14, 1915	4/17 0 (B. avisepticus) 1:40 (B. ty- phosus)		24
					48
600	Hemorrhagic-septi- cemic vaccine 1 injection	Feb. 17, 1916	>1:200 (Iowa I) 0 (B. ty- phosus 49)	>0.003 (B. bipolaris 49)	24
					48
611	B. abortus toxin 2 injections (6 c.c.)	March 1, 1916	1:200 (B. abortus- equi)	0.02 (B. abortus- equi)	24
					48
					72
646	Control		0 (Olsen)	0.05 (50% with B. typhosus)	24
					48
647	Control		0 (Olsen)	0.1 (50%)	24
					48
648	Control		1:20 (Olsen)	0.1 (50%)	24
					48
					72

TABLE 3—Continued

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN PREPARATIONS (GROUP 2)

DATE OF TESTS: APRIL 28, 1916

Typhoidin 15		Control-Powder 15 C 1:100	Fowl-Ty-phoidin 4 1:100	Control-Powder 1:10	Agar (0.5%)	Abortin 1:100	Remarks
1:50	1:100						
(cm.) 1.8 x 1.8, atypical 2 x 2, atypical	(cm.) 0.9 x 0.9 0.8 x 0.8	(cm.) N. P.	(cm.) 2.2 x 2.2 2 x 2	(cm.) 0.9 x 0.9 0.5 x 0.5	(cm.) 0.8 x 0.8 0.5 x 0.5, whitish nodule	(cm.)	
0.9 x 0.9, whitish 0.5 x 0.5, nodule	N. P. N. P.	Red spot Red spot	1.5 x 1.5, R. I. 1.4 x 1.5, R. I.	0.5 x 0.5, yellow Red spot	N. P. N. P.		
2 x 2, I. R. 1.6 x 1.6, I. R.	2 x 2, I. R. 1.5 x 1.5, I. R.	Red spot N. P.	Red spot Red spot	1.8 x 1.8 1.4 x 1.4	N. P. 0.6 x 0.6	N. P. N. P.	Re-injected on April 25. Car- rier
Red area 1 x 1, red area 0.8 x 0.8	Red spot Red spot Red spot	N. P. N. P. N. P.	0.9 x 0.9 1.2 x 1.2 1.8 x 1.8	0.5 x 0.5 Red spot Red spot	0.9 x 0.9 0.9 x 0.9 1 x 1		Re-injected on April 25. Died from typhoid relapse
Red area 2.2 x 2.2 1.4 x 1.4	Red area N. P. N. P.	N. P. N. P. N. P.	Red spot Red spot N. P.	Red spot N. P. N. P.	Small nod. Small nod. N. P.		Re-injected on April 25. Not a carrier
1.5 x 1.5 Red spot	1.4 x 1.4 1.4 x 1.4	Red spot N. P.	1.5, D. R. A. 1.6 x 1.6, D. R. A.	0.5 x 0.5 Red spot	Small nodule Small nodule	N. P. N. P.	
N. P. N. P.	N. P. N. P.	N. P. N. P.	N. P. N. P.	N. P. N. P.	N. P. N. P.	N. P. N. P.	Clinically sick, emaciated
Red area 1.2 x 1.2, whitish	Red area 0.6 x 0.6, slight reddening	N. P. N. P.	1.1 x 1.1 1.1 x 1.1	N. P. Red spot	N. P. N. P.	N. P. N. P.	
2 x 2 1.7 x 1.7	1.9 x 1.9 0.8 x 0.8	0.5 x 0.5 Red spot	1.4 x 1.4 1.7 x 1.7	Red spot Red area	0.9 x 0.9 Red nodule	0.9 x 0.9, red area Nodule	
0.5 x 0.5, D. R. A. Nodule	0.5 x 0.5, red area Nodule	Red spot N. P.	0.2 x 0.2, nodule N. P.	Red area Small nodule	N. P. N. P.	N. P. N. P.	
D. R. A. 0.1 x 0.1, red spot	D. R. A. 0.1 x 0.1, red spot	N. P. N. P.	1.8 x 1.8 1.3 x 1.3	Red area Red spot	0.9 x 0.9, R. I. 0.6 x 0.6	N. P. N. P.	
Red spot 1.4 x 1.4, atypical Red nodule	N. P. N. P. N. P.	N. P. N. P. N. P.	0.5 x 0.5 0.5 x 0.5 Red spot	0.8 x 0.8 0.5 x 0.5 Red spot	0.5 x 0.5 0.5 x 0.5 N. P.	N. P. N. P. N. P.	
N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	0.3 x 0.3 N. P. 0.5 x 0.5 N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	
Red area 1.5 x 1.5 N. P.	Red spot 1.1 x 1.1 Red spot	N. P. N. P. N. P.	0.4 x 0.4 0.4 x 0.4 N. P.	0.4 x 0.4 Red spot N. P.	N. P. N. P. N. P.	N. P. N. P. N. P.	

typhoid bacillus, served as controls for the typhoidin preparation. One hundred and sixty-one days had elapsed since the last inoculation, and their immunity as judged from the amount of complement-fixing antibodies present in the serum, was correspondingly slight; the agglutinins, however, were still present in fair amounts.

The other rabbits had been immunized with *B. bipolaris-septicus* (447, 463, 495, 600), a yeast isolated from sputum (484), dog serum (313, 316), *B. pullorum* (492), and *B. abortus-equinarius* (611). For details regarding the immunization and the time interval following the last injection, the reader is referred to Table 3.

The glycerin extract of the typhoid bacillus, Typhoidin 15, with an antigenic value of 5 e.u., was a low-grade preparation obtained by growing the strain Olsen in a glycerin veal potato broth for 24 days. The unfiltered culture, which showed a heavy sediment, was concentrated to one-tenth its volume and precipitated as usual. The powder possessed a grayish tinge, was granular, and dissolved only with difficulty.

Fowl-typhoidin 4 was a preparation made with Witte's peptone, which had been grown for 5 days at 37 C.; its value was 20 e.u. per 0.001 gm. of powder. The abortin was a glycerin extract of cultures of *B. abortus* from 10 to 12 weeks old, and had been prepared in the usual manner. The colloid was a 0.5% solution of agar in salt solution, sterilized at 120 C.

The results of this experiment are described in detail in Table 3. Typhoidin 15 in amount of 0.001 gm. proved to be a weak antigenic substance. Only Rabbit 328 of 3 typhoid-immune animals (328, 329, 330) developed readable reactions; 2 other rabbits (447 and 492), immune to *B. pullorum* and *B. bipolaris-septicus*, respectively, gave positive typhoidin tests however. The powder was not absolutely inert in one normal rabbit (648)—one of those animals showing an individual hypersensitiveness to the toxic properties of the broth constituents.

In the amount of 0.02 gm., the powder produced specific reactions in 2 typhoid-immune rabbits (328 and 330), but in 2 rabbits (313 and 611) not immunized against the typhoid bacillus, atypical areolae appeared which deserve further description and discussion. A small hyperemic, slightly warm area developed in the first 24 hours; induration was slight, or of such degree that it was only recognized with difficulty. Inside this area was a whitish center, which enlarged to a small nodule in the next 48 hours. Similar reactions were noted in some rabbits at the site of injection of Broth-control 4, which was used in an exceptionally high concentration. It is quite evident that most of the rabbits which reacted to the agar solution reacted also to these concentrated powder suspensions, which were rather insoluble in salt solution, turbid and milky, and had all the properties of a colloidal solution. It is possible that these atypical reactions are the

result of a mechanism which Stokes has discussed and explained on the basis of his experimental work. Reasoning from Jobling's and Petersen's¹¹ conception of anaphylaxis, Stokes has formulated a theory that the antiferment-absorbing effect of colloids is responsible for a local disturbance of the protease balance with production of toxins which, in turn, cause the vascular disturbances characteristic of an intracutaneous reaction in man.

The rabbits which developed these nonspecific skin reactions developed good typical areolae also with the fowl typhoidin. We suspect, therefore, that these cutaneous reactions, typical as well as atypical, are the result of individual conditions, in part due perhaps to the immunization and therefore similar to those noted and described in previous experiments.

For the first time we noticed that a weak typhoidin preparation failed to produce specific skin reactions in some properly immunized animals. In Rabbit 329 not even 0.02 gm., and in 330 only this amount, called forth a typical reaction. According to the agglutination titer these rabbits had developed about the same amounts of immune substances. It is of interest to note at this time that in a subsequent immunity test, Rabbit 328, which gave a well-marked typhoidin reaction, became a carrier (gall-bladder). Rabbit 330 resisted the infection and Rabbit 329, which reacted only to fowl typhoidin, succumbed in 23 days after the intravenous injection of living typhoid bacilli, to an acute typhoid relapse.

Fowl-typhoidin 4, which according to previous experiments called forth specific skin reactions in typhoid-immune rabbits, produced in the test under consideration a typical and persistent areola in 1 rabbit only (329). A specific reaction was also noted in Rabbit 492. The nonspecific response in 313, 316, 484, and 600 is, as already pointed out, the result either of intensive sensitization to bacterial proteins, or of metabolic changes following the injection of foreign proteins (313, 316).

The positive reactions in Rabbits 313 and 316 are the most difficult ones to explain. It is not unlikely that 27.5 c.c. of dog serum produced nonspecific changes and conditions of hypersusceptibility to bacterial extracts which are perhaps similar to those produced in hyperimmunization with bacteria. The inflammatory character was well marked in the reaction to fowl typhoidin, and at first it was thought that these

¹¹ Jour. Exper. Med., 1915, 21, p. 239; 1914, 20, p. 37.

skin reactions were due to the primary toxicity of the powder, or to traumatism. But persistence of the reaction up to the 48th hour, and slight or negative results in the control rabbits, recorded in Tables 1 and 3, do not support this contention.

The abortin in dose of 0.001 gm. was inert in all rabbits; some indications of a reaction were seen in the hyperimmune rabbit 492, which responded, as stated, to all the bacterial proteins inoculated.

Group 1 permits certain conclusions. A 24-day-old glycerin-potato-broth culture of *B. typhosus* produces a typhoidin which has low antigenic value, and which accordingly is only active in dilutions of 0.02 gm. On account of its insolubility in salt solution, it may act on intracutaneous injection as other colloids like agar, giving rise to atypical reactions. Rabbits immunized with bacterial or yeast protein and apparently also those immunized with animal (dog) protein, otherwise in good health, acquire cutaneous hypersusceptibility to glycerin extracts of bacterial broth cultures. The intensity of reaction is directly influenced by the amount of protein introduced. Hyperimmune animals which have recovered from an infection (447) will react with every glycerin extract used in the experiment.

EXPERIMENT-GROUP 3

Inasmuch as it was not quite clear what caused these nonspecific reactions, which had occurred in every experiment thus far conducted, and inasmuch as our interpretation of them was based on the idea of sensitization, we conducted some experiments in which the disturbing factor of broth constituents in the typhoidin powder was eliminated. Typhoidin powders carefully redissolved, filtered, and again precipitated, constituted one antigen, and an autolysate of the typhoid bacillus grown on agar, the other. The latter represented a pure preparation of the various typhoproteins of the bacillus.

At present we shall report one experiment (3), and we hope to show in the near future that the suggestion therein recorded will be of value in the discussion of sensitization in general.

Thirteen rabbits previously injected by various methods with animal and bacterial proteins or living bacteria, were used in this experiment. Five rabbits (749 to 753), corresponding in age and size to the test rabbits, served as controls. Rabbits 691 and 692 had been immunized, by a rapid method with 5 injections, against 2 different strains of *B. typhosus*. Forty days after the last injection they had received another dose of living typhoid bacilli. They were tested 24 days after the last injection.

Rabbits 716, 717, and 719 had been immunized in similar manner with typhoid, and recently isolated paratyphoid-A organisms, respectively, and were tested 14 days after the last injection. One rabbit (711) had been immunized with living paradysenteriae bacilli (Strain 9). It had received 5 intravenous injections and was tested 7 days after the last injection.

About 14 to 48 days prior to the skin tests, several rabbits (703, 704, 705, 706, 718, 741) had been injected directly in the gallbladder with small doses of living dysentery, paradysentery, and typhoid bacteria. Most of these animals showed a slight response to the infection as judged by the serum immune bodies.

Two rabbits (620 and 695) had received several intravenous injections of sheep cells and had produced a low-grade hemolysin. A final injection had been given 3 days before the test. The typhoidin preparations tested in this experiment were, for reasons already stated, different from those hitherto described.

Preparation Ia.—This was a 5-day-old glycerin rabbit-broth-culture extract of an old typhoidin strain which had been passed several times through the gallbladder of a rabbit.

The concentrated extract, before precipitation, was filtered through Chardin paper. The final product contained 20 e.u. per 0.001 gm. and, according to Chart 1 in our 1st paper, it was a good antigen.

Preparation Ib.—This was the purified precipitate of Ia. The powder was 3 times redissolved in salt solution and again precipitated with absolute alcohol. A somewhat more concentrated antigen was thus obtained, the antigenic value of the powder being 33.3 e.u. per 0.001 gm.

Preparation IIa.—This was prepared with a recently isolated strain of *B. typhosus*, but was unfiltered. It had an antigenic value of 20 e.u. per 0.001 gm. and was a poor antigen. As controls for the rabbit-broth and peptone contents, the concentrated and precipitated rabbit broth was used.

Preparation V.—This was obtained as follows: The 48-hour-old agar surface growth of several Blake bottles of the same strain of *B. typhosus* as used in Preparation IIa, was suspended in distilled water, shaken for one-half hour, and heated for 1 hour at 60 C. The autolysate was precipitated with alcohol and dried in vacuo to a scaly mass. This typhoprotein was soluble with difficulty in carbolized salt solution, and represented in the dilution of 1:100 a thick milky suspension. In a dilution of 1:1000 it was turbid and, on standing, a whitish débris settled out.

Inoculated intravenously into a rabbit, it caused, in less than 24 hours, acute intoxication in dose of 0.1 gm.; small doses like 0.01 gm. provoked loss of weight (100 to 200 gm.) and temporary illness. As a whole, however, its toxic effect on the animals by intravenous injection was less compared with that of some of the glycerin extracts. In amount of 0.001 gm. subcutaneous injections of this concentrated bacterioproduct caused abscess-formation and induration. The preparation was exceedingly antigenic, containing 333 e.u. per 0.001 gm.

Tuberculin Bovine C.—This was the alcoholic precipitate of an old concentrated, but not filtered, culture of a bovine type of tubercle bacillus. To determine the effect of the tubercle protein the powder was used in the concentration of 0.02 gm.

Pork Protein.—This was a 10% salt solution of pork protein prepared from the alcoholic precipitate obtained according to the method of Wodenhouse.¹²

¹² Boston Med. and Surg. Jour., 1916, 175, p. 195.

TABLE 4
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN (GROUP 3)

Rabbit	Immunization	Last Infection	Serologic Tests		Hours	Typhoidin Ia 1:100	Typhoidin Ia 1:100
			Agglutination	Complement-Fixation			
620	Sheep hemolysin	Oct. 7, 1915	1:20 (H-125)		24	(cm.) 0.6 x 0.6, not in- durated	(cm.) 0.8 x 0.8, not in- durated
					48	0.2 x 0.2, nodule	0.2 x 0.2, nodule
					120
691	B. typhosus Nelson 6 injections	Sept. 16, 1916	10/7 1:6000	(0.0005) 0.001	24	1.4 x 1.4, well-defined nodule	0.9 x 0.9, well-defined nodule
					48	1.1 x 1.1	0.9 x 0.9, nodule
					120
692	B. typhosus 323 6 injections	Sept. 16, 1916	10/7 1:6000	>0.0005 0.002	24	1.3 x 1.3, R. I. not well defined	1.4 x 1.4, R. I., W. D.
					48	0.5 x 0.5, well-defined Nodule	1.2 x 1.2, W. D. Nodule
					120
695	Sheep hemolysin	Oct. 7, 1916	1:20 (H-125)		24	Red spot	Red spot
					48	Red spot, 0.5 x 0.5	Red spot
					120
703	B. paratyphosus 9 Gallbladder- injection	Sept. 2, 1916	1:100 to 1:200		24	Red spot	1 x 1, red area
					48	Red spot	Red spot
704	B. dysenteriae Shiga Do. Gallbladder- injection	Sept. 2, 1916	1:80		24	Rde spot	Red spot
					48	Red spot	Red spot
705	B. paratyphosus A, Fraser Gallbladder- injection	Sept. 2, 1916	10/7 1:2000	0.03	24	Red spot	1.4 x 1.4, W. D.
					48	Red spot	1.5 x 1.5, W. D.
					120
706	B. dysenteriae Mt. Desert Gallbladder- injection	Sept. 2, 1916	1:60		24	1.6 x 1.6, D. R.	1.1 x 1.1
					48	Red area	Red area
					120
711	B. dysenteriae Honolulu 9 intravenously		1:2000 —1:6000	0.001	24	Red spot	1 x 1, no induration, whitish patch
					48	Whitish area 0.5 x 0.5
					120
716	B. typhosus Mexico 6 injections	Sept. 26, 1916	1:4000	>0.0005	24	1.8 x 1.8, yellow center	1.9 x 1.9, R. I.
					48	1.1 x 1.1, nodule	1.4 x 1.4, R. I.
					120	0.5 nodule	Nodule

TABLE 4—Continued

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN (GROUP 3)

Typhoidin 1-b 1:100	Control- Powder 1:25	Typhoidin V		Tuberculin B. C. 1:50	Pork Protein 1:10	Remarks
		1:100	1:1000			
(cm.) 1 x 1, not in- durated 0.2 x 0.2, nodule	(cm.) 0.2 x 0.2 N. P.	(cm.) 2 x 2, R. I. 1.4 x 1.4, R. I. nodule	(cm.) 0.3 x 0.3, R. I. 0.1 x 0.1, nodule	(cm.) 0.8 x 0.8, R. I. 0.3 x 0.3, nodule nodule	(cm.) N. P. N. P.	
1.4 x 1.4, well-defined nodule 1.4 x 1.4, well-defined nodule	0.8 x 0.8 N. P.	1.5 x 1.5, well-defined nodule 1.4 x 1.4, well-defined nodule Nodule	0.9 x 0.9, nodule 0.5 x 0.5, nodule	0.8 x 0.8, raised nodule 1 x 1 nodule size of a pea	N. P. N. P.	
0.6 x 0.6, well-defined nodule 0.7 x 0.7, well-defined nodule Nodule	0.5 x 0.5, not well defined N. P.	1.8 x 1.8, very well defined 1.8 x 1.8, R. I. Nodule	0.7 x 0.7, W. D. 0.9 x 0.9, W. D. Nodule	0.5 x 0.5, raised induration 0.7 x 0.7, well-raised nodule Nodule size of a pea	N. P. N. P.	
0.4 x 0.4 1.2 x 1.2	0.6 x 0.6 N. P.	1 x 1 1.9 x 1.9, R. I. Nodule	0.6 x 0.6 N. P.	0.7 x 0.7 0.2 x 0.2	N. P. N. P.	
N. P.	N. P.	1.7 x 1.7	1.2 x 1.2	0.8 x 0.8	N. P.	
N. P.	N. P.	1.8 x 1.8	0.8 x 0.8	0.5 x 0.5	N. P.	
1.5 x 1.5, R. I. 1.4 x 1.4, R. I.	0.5 x 0.5 N. P.	1.8 x 1.8, R. I. 1.8 x 1.8	1 x 1, R. I. 0.2 x 0.2, red spot	0.5 x 0.5 0.8 x 0.8, raised nod.	N. P. N. P.	
1.7 x 1.7, R. I. 1.2 x 1.2, I. R.	0.8 x 0.8 N. P.	2 x 2, R. I. 1.5 x 1.5, R. I. Nodule	N. P. N. P.	0.5 x 0.5 N. P.	0.3 x 0.8 N. P.	
1.6 x 1.6 Red area, no induration	0.8 x 0.8 N. P.	1.8 x 1.8, R. I. 1.4 x 1.4, R. I. Nodule	1.5 x 1.5, R. I. 1.1 x 1.1, R. I.	0.5 x 0.5 0.5 x 0.5 Nodule small	N. P. N. P.	
0.8 x 0.8, Red area, no induration 0.9 x 0.9, Red area, no induration	0.5 x 0.5 N. P.	1.8 x 1.8, R. I. 1.8 x 1.2, R. I. Pin-head nodule	0.6 x 0.6, red N. P.	0.5 x 0.5 0.4 x 0.4 Pin-head nodule		
1.3 x 1.3, yellow center 0.9 x 0.9	0.3 x 0.3 N. P.	1.8 x 2.8, yellow center 2.1 x 2.1, R. I. 1.5 x 1.5, Nodule	0.9 x 0.9 1.3 x 1.3 Nodule	1 x 1, R. I. 0.5 x 0.5, R. I. 0.5, nodule	N. P. N. P.	

TABLE 4—Continued
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN (GROUP 3)

Rabbit	Immunization	Last Infection	Serologic Tests		Hours	Typhoidin IIa 1:100	Typhoidin Ia 1:100
			Agglutination	Complement-Fixation			
717	B. paratyphosus A, Fraser 5 injections	Sept. 26, 1916	1:20,000	0.001	24	(cm.) 1.9 x 1.9, R. I.	(cm.) 1.5 x 1.5, R. I.
					48	1.5 x 1.5, R. I.	1.2 x 1.2, R. I.
					120	Nodule	Nodule
718	B. typhosus Kearney Gallbladder-injection	Sept. 16, 1916	1:2000	0.01 —0.0075	24	1 x 1, R. I.	1.1 x 1.1, R. I.
					48	0.7 x 0.7, nodule	0.7 x 0.7, R. I.
					120
719	B. paratyphosus A, Vaughn	Sept. 16, 1916	1:10,000	0.0005	24	0.8 x 0.8, W. D.	1.1 x 1.1, red
					48	1.8 x 1.8, red area	1 x 1
					120
741	B. paradysenteriae Napa Gallbladder-injection	Sept. 26, 1916	1:200 —400		24	1.5 x 1.8, red area	0.7 x 0.7
					48	0.6 x 0.6, nodule	0.6 x 0.6, nodule raised
					120
749	Control		1:100		24	1.3 x 1.3, red area	1.2 x 1.2, red area
					48	1.4 x 1.4, red area	0.2 x 0.2, nodule
750	Control		1:40		24	1 x 1, red	1.5 x 1.5, red area
					48	N. P.	N. P.
751	Control		1:20		24	1.3 x 1.3, red	0.8 x 0.8, R. I.
					48	0.9 x 0.9, nodule	0.4 x 0.4, R. I.
					120
752	Control		1:20		24	N. P.	N. P.
					48	N. P.	N. P.
753	Control		1:20		24	N. P.	N. P.
					48	N. P.	N. P.

The results of this test are summarized in Table 4. The cutaneous response of the rabbits to the glycerin extracts of *B. typhosus* and that to the pure typhoprotein in this experiment were visibly not of the same intensity, and the reactions did not run parallel in the different animals. On account of the marked effect of this latter preparation on the control animals, the results obtained with Typhoidin V in a dilution of 1:100 must be excluded from consideration. The pure typhoprotein in dilution of 1:1000 (0.0001 gm. = 0.1 mg.) is therefore more suitable for our comparative studies.

TABLE 4—Continued

RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN (GROUP 3)

Typhoidin I-b 1:100	Control- Powder 1:25	Typhoidin V		Tuberculin B. C. 1:50	Pork Protein 1:10	Remarks
		1:100	1:1000			
(cm.) 1.2 x 1.2, R. I. 0.8 x 0.8, R. I. Nodule	(cm.) 0.8 x 0.8, R. I. 0.5 x 0.5, R. I.	(cm.) 2.5 x 1.9, R. I. 2.4 x 2.4 Nodule	(cm.) Diffuse red area 0.6 x 0.6	(cm.) Diffuse red- dish area N. P. Nodule	(cm.) N. P. N. P.	Lost in weight
1.2 x 1.2, R. I. 0.4 x 0.4, R. I.	N. P. N. P.	1.7 x 1.7 1.1 x 1.1	1.2 x 1.2, red 1 x 1	1.1 x 1.1 0.4 x 0.4 Nodule	Suppurative cholecystitis Nov. 18, 1916
1 x 1, R. I. 0.6 x 0.6	0.5 x 0.5 0.4 x 0.4	2.2 x 2.2, D. O. 2.1 x 2.1 Nodule	2.5 x 2.5, edema 1.5 x 1.5	0.7 x 0.7 0.7 x 0.7 Nodule	N. P. N. P.	
1.8 x 1, R. I., edema 0.6 x 0.6, raised nod.	0.2 x 0.2 N. P.	About 4.5 x 2.8, D. O.	About 4.5 x 2.8, D. O.	0.8 x 0.8 0.4 x 0.4	N. P. N. P.	
0.7 x 0.7 0.2 x 0.2, nodule	N. P. N. P.	1.6 x 1.6, R. I. 1.9 x 1.9, R. I., nodule 2 x 2	0.4 x 0.4 N. P.	0.4 x 0.4 0.3 x 0.3	N. P. N. P.	
0.8 x 0.8, red N. P.	N. P. N. P.	1.2 x 1.2 1 x 1, nodule	N. P. N. P.	0.6 x 0.6, red 0.5 x 0.5, nodule		
0.9 x 0.9, red 0.8 x 0.8, red	0.6 x 0.6 N. P.	3.8 x 3.8, D. O. 2.8 x 4.8, D. O.	3.8 x 3.8, D. O. 2.8 x 4.8, D. O.	0.8 x 0.8 0.4 x 0.4	N. P. N. P.	
N. P. N. P.	N. P. N. P.	2.1 x 1.6 1.7 x 1.7	0.6 x 0.6 0.2 x 0.2	0.5 x 0.5 0.5 x 0.5	N. P. N. P.	
N. P. N. P.	N. P. N. P.	1.5 x 1.5 1.4 x 1.4	0.5 x 0.5 0.5 x 0.5	0.5 x 0.5 0.3 x 0.3	N. P. N. P.	

Of 3 rabbits (691, 692, and 716) immunized against *B. typhosus*, only 1 rabbit (716) gave a well-defined fair-sized reaction after 24 hours, which persisted for 2 or 3 days. Animals 691 and 692 gave, with typhoprotein, reactions which differed very little from those of the controls, but they reacted distinctly to one or two of the glycerin extracts. The crude unfiltered (II^a) and also the unpurified (I^a) preparations were visibly more active, but correspondingly irritant to the skin of several control rabbits (749, 750, 751). These reactions are in many respects similar to those in Experiment-group 4, soon to be discussed.

There is not much difference in action between the typhoidin preparations I^a and I^b. The purified extract I^b provoked somewhat less intensive reactions than I^a. In considering the typhoidin I^a as the best of the glycerin-extract preparations, we found that Rabbit 692 gave a marked persistent reaction, which was surpassed in intensity, however, by that of the more recently immunized rabbit, 716.

Quite in accord with our previous observations was the finding that rabbits highly immunized with bacteria other than typhoid — for example, in this experiment *B. paratyphosus* A (Rabbits 717 and 719) — will give strong reactions to glycerin extracts and, in a slight but definite degree (719), to typhoprotein also. The only rabbit (711) intensively immunized with paratyphoid organisms reacted like the controls, both to extracts and to typhoprotein.

The skin reactions of the rabbits infected by gallbladder-inoculations are not conclusive. Rabbits 705 and 718 with a fair degree of serum immunity gave well-defined, well-indurated reactions to the filtered glycerin extract; the typhoid-infected rabbit (718) reacted positively also to typhoprotein.

The dysentery rabbits (703, 704, 706, 741) reacted slightly, if at all, to the nonpurified glycerin extracts, but gave well-defined and marked reactions to the typhoprotein and the purified typhoidin I^b. Most of the areolae were well defined and persisted in some rabbits for at least 48 hours. These rabbits possessed a slight degree of immunity because the dysentery infection remained in the biliary passages and very little bacterial protein was probably absorbed to produce the condition of generalized nonspecific hypersensitiveness to bacterial glycerin extracts so commonly observed throughout our experiments in highly immunized animals.

In our opinion, it is not a mere coincidence that in these paratyphoid-infected rabbits the reactions to typhoprotein ran parallel with those to the concentrated and purified typhoidin I^b. This observation supports our views that this cutaneous hypersensitiveness is due to substances of the bacterial body and not to metabolic products developed in the broth by the growth of the microorganism. In the purified extract powders and the typhoprotein, these substances are more concentrated than in the crude and unfiltered preparations.

The bovine tuberculin B. C. produced reactions which can be compared with those produced by other colloidal suspensions. As a rule, the rabbits which reacted well to unfiltered glycerin extracts also pro-

duced areolae with tuberculin; for example, 716, 718, and 691. In the same animals the reaction persisted for many days in the form of a small aseptic tuberculoma. The high concentration of the tuberculo-protein is probably in part responsible for these reactions; they lack specificity, for some of the control rabbits reacted similarly, tho not so intensely.

Pork protein was inactive in all the rabbits tested.

With Group 3 the results were as follows: A filtered glycerin rabbit-culture extract of *B. typhosus* called forth distinct reactions in typhoid- and paratyphoid-immune rabbits; as a rule, the areolae were smaller than those noted for the unfiltered preparation. Filtered typhoidin powder is also less irritant to normal healthy rabbits than the unfiltered product. The purified and more concentrated typhoidin is not very irritant to normal rabbits; it gives smaller reactions and behaves somewhat as the pure typhoprotein does. This again indicates that the amount of specific substance demonstrable by complement-fixation test, or the so-called antigenic value of a typhoidin preparation, does not always indicate the suitability of the powder as an antigen for skin tests. This is particularly true for the typhoprotein with a value of 333 c. u. per milligram. The skin reactions to this highly potent preparation in dilutions inert for control animals, were small, sometimes indistinct, and therefore frequently inconclusive. On the other hand, concentrated suspensions of typhoprotein were nonspecific. On account of their high concentration in bacterial protein, they acted as severe irritants and produced extensive pyogenic reactions in immunized and normal rabbits.

It is quite apparent that pure bacterial proteins are capable of producing skin reactions in immune or infected animals, just as Deehan¹² has observed in typhoid cases in man. The results thus far available from this one test, indicate only that these reactions can have a certain degree of specificity as judged by the intensity of the reaction in typhoid- or paratyphoid-immune rabbits. In choosing another, perhaps more purified, preparation, it may be possible to obtain even more specific reactions which would be conclusive evidence that the cutaneous hypersensitiveness is due to sensitization with some simple or complex element of the typhoprotein. From a diagnostic viewpoint it seems paradoxical, however, that in the rabbit the pure bacterial protein of the typhoid bacillus should be less suitable for cutaneous

¹² Univ. of Penn Med Bull., 1909, 22, p. 103.

tests than the purified glycerin extract. Previous observations have shown that suspensions of tubercle bacilli are better antigens than tuberculin for diagnostic skin tests in tubercular rabbits. Additional experiments, now in progress, will throw more light on this interesting finding.

From the animals of Experiment-group 3 no further information can be drawn concerning the nature of the nonspecific reactions in highly immunized rabbits; it is however quite apparent that the pure typhoprotein which does not contain substances of the broth, gives reactions with dysentery-infected or with immune rabbits. These reactions are, as already described, paralleled by those to the purified typhoidin 1^b. The latter preparation proved to be nontoxic in some of the rabbit-inoculation experiments, 0.2 gm. inoculated intravenously producing slight loss in weight and yet giving rise to considerable amounts of agglutinins (see Chart 1, 1st paper). The reactions cannot, therefore, be ascribed to the action of the endotoxin. They suggest, however, a condition of cutaneous hypersensitiveness, which is probably a form of sensitization to certain elements of the bacterial proteins common to most microorganisms.

The evidence collected in Group 3 did not appear sufficient to justify the conclusion that the cutaneous typhoidin reaction is due to sensitization, and we attempted, therefore, the passive transfer of the hypersensitiveness.

Gay and Claypole⁴ reported experiments in which they succeeded in transferring the susceptibility to typhoidin from an immune to a normal animal. They proved that the skin reaction is the result of an interaction of antigen and antibodies. We repeated these experiments successfully in 2 exactly similar experiments by inoculating 20 c.c. of rabbit typhoid-immune serum into the peritoneal cavity of a normal rabbit and testing the latter with typhoidin and similar preparations 24 hours later.

The cutaneous hypersensitiveness was present, not only to the typhoid, but also to the fowl-typhoid antigen. The reactions to extracts of *B. dysenteriae* were inconclusive. Further experiments along the same lines are in progress with the main object of finding out whether specific hypersensitiveness alone, or together with the non-specific factors, is transferable from immune, hyperimmune, or infected rabbits to normal animals of the same species, and also to guinea-pigs.

EXPERIMENT-GROUP 4

In our first communication we called attention to the accidental observation that normal healthy rabbits which had served as controls in our typhoidin tests would give positive skin reactions when retested, within 1 to 2 months, on another portion of the skin. This acquired cutaneous hypersensitiveness was present for at least 2 months in some rabbits. We suspect that the retests were in part responsible for renewing the hypersusceptibility to typhoidin. Group 4 illustrates some of the reactions recorded.

Four rabbits (607, 608, 609, and 640) which had served as controls in Group 1, Series 1 and 2, and in Group 1, 3rd paper, were tested along with a number of immune and normal rabbits which were being used in connection with some other experiments. These rabbits gave, on the first test, very slight or negative reactions. In neither immune nor normal animal did the diameter of the areola exceed 0.5 cm. in the first 24 hours. Only in Rabbit 609 had the agglutination titer changed between the 1st and 2nd tests. Specific complement-fixation antibodies could not be demonstrated in either rabbit. The typhoidin preparations P.C. and E. used in the group, were suitable antigens and have been fully considered in the experiments in which these rabbits were included.

In Tables 5 and 6 the results are summarized. Rabbits 607 and 608 reacted to the intracutaneous typhoidin test in the same manner as immunized or recovered animals. The reactions were well marked and correspondingly persistent for 48 hours. It is rather interesting to note that Rabbit 608 — which received quantitatively more bacterial extracts at the 1st test on February 23 — showed not only better reactions with typhoidin than did Rabbit 607, but also noteworthy group reactions with paratyphoidin-A and -B, and B.-coli extracts. The few milligrams of antigen were apparently sufficient to produce general cutaneous hypersensitiveness.

In a similar manner, Rabbits 609 and 640 reacted to Typhoidin E, and the other extracts used in Group 1, Series 2. The results are not so striking, on account of the different character of the typhoidin preparations used in this experiment.

In comparison with the controls 662 and 663 (Table 2), the sensitized control rabbits 609 and 640 gave good reactions to broth typhoidin. In every respect the areolae appeared to be identical in character with those on the typhoid-immune rabbits tested simultaneously.

The experience summarized in Group 4 furnishes the proof that cutaneous hypersensitiveness to typhoidin is readily acquired by rabbits.

¹⁴ Compt. rend. Soc. de biol., 1907, 63, p. 296.

TABLE 5
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN (GROUP 4)
DATE OF TESTS: APRIL 10, 1916

Rabbit	Previous Test	Serologic Tests, April 10, 1916		Hours
		Agglutina- tion	Complement- Fixation	
607	Control Feb. 23, 1916 Typhoidin Kuhn Fowl Typhoidin, A. P. Smith Staphylococin	1:60	0.05 (50%)	16
				24
				48
				72
608	Control Feb. 23, 1916 Typhoidin, Rawlings Dysentery Do. B. coli	1:20	0.1 (50%)	16
				24
				48
				72

Calmette, Breton, and Petit¹⁴ reported that nontubercular rabbits would give positive ophthalmic reactions after the subcutaneous injection of tuberculin; these authors explain this observation as a phenomenon of local sensitization. Similar conditions apparently develop as a result of intravenous or subcutaneous injections of typhoidin. In 3 experiments we could show that in this manner the application of typhoidin preparations in the amounts of 0.01 gm. produced cutaneous hypersensitiveness to the same or to similar bacterial extracts. One of these experiments is illustrated in Table 7.

The experimental data thus far collected, strongly support the conception that the typhoidin reaction is in all probability the result of a process of sensitization. One must reserve for further investigations the determination of the elements of the typhoid bacillus which are responsible for this sensitization, and great difficulty is to be

TABLE 6
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN (GROUP 4)
DATE OF TESTS: APRIL 24, 1916

Rabbit	Previous Test	Serologic Tests, April 24, 1916		Hours
		Agglutina- tion	Complement- Fixation	
609	Control Feb. 23, 1916 Typhoidin 3 Staphylococin 4 Paratyphoidin	1:40-1:100	0.05 (50%)	24
				48
640	Control April 10, 1916 Fowl typhoidin Staphylococin Paratyphoidin B Dysentery Do.	1:40-1:100 (Fowl typhoidin) (1:1000)	0	24
				48

TABLE 5—*Continued*RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN (GROUP 4)
DATE OF TESTS: APRIL 10, 1916

Typhoidin P. C.		B. Paratyphoid A 1:100	B. Coli	Paratyphoidin B 1:100	Control Powder
1:50	1:100				
(cm.) 1.6 x 1.6 1.9 x 1.9 2.2 x 2.2 N. P.	(cm.) Red area Red spot N. P. N. P.	(cm.) Red area 1.6 x 1.6 Red area Red spot	(cm.) Diff. red area Diff. red area 0.2 x 0.2	(cm.) Red spot Red spot N. P. N. P.	(cm.) 1.4 x 1.4 Red area N. P. N. P.
1.4 x 1.4 2 x 2 1.8 x 1.8	1 x 1 1.6 x 1.6 1 x 1	1.5 x 1.5 1.4 x 1.4 1 x 1	1 x 1 0.9 x 0.9 1 x 1	Red area Diff. red area 1.7 x 1.7	N. P. Red spot Red spot

expected as long as we know so little about the nature of bacterial anaphylaxis in general.

From a diagnostic viewpoint it is important to remember that cutaneous hypersensitiveness is readily acquired by rabbits. This fact deprived us of the means by which we originally intended to follow the gradual disappearance of the hypersusceptibility in its relation to the resistance in an infection. Thus far it appears that the rabbit is unsuitable for the study of cutaneous sensitization.

DISCUSSION

The variability and suitability of the various glycerin extracts of typhoid and other bacterial cultures have been discussed in connection with each experiment group, and it has been pointed out that, as a rule, rabbits immunized against *B. typhosus* will give well-defined positive

TABLE 6—*Continued*RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOIDIN (GROUP 4)
DATE OF TESTS: APRIL 24, 1916

Typhoidin E		Control Powder	Staphylococci	Paratyphoidin A 1:100	Paratyphoidin B 1:100
1:100	Undiluted Broth				
(cm.) 1 x 1 0.9 x 0.9	(cm.) 1.2 x 1.2 1 x 1	(cm.) N. P. N. P.	(cm.) N. P. N. P.	(cm.) N. P. N. P.	(cm.) N. P. N. P.
1.6 x 1.6 1.4 x 1.4	1.6 x 1.6 1.8 x 1.8	0.5 x 0.5 Red spot	Red spot 0.5 x 0.5	N. P. 0.8 x 0.8	1.2 x 1.9 1.4 x 1.4

TABLE 7
RESULTS OF INTRACUTANEOUS TESTS WITH TYPHOPROTEIN

Rabbit	Injection	Agglu- tin- ins	Hours	Typho- protein 323	Typho- protein Nelson	B.-Coli Protein	Army Vaccine	Staphylo- coccus Protein
742	Typhoidin Ia 40 days previ- ous to test	1:200	24	1.5 x 1.4, R. I.	0.8 x 0.8	1.3 x 1.3	0.6 x 0.6	0.6 x 0.6
			48	1.1 x 1.1, R. I.	N. P.	0.5 x 0.5	0.2 x 0.2	0.4 x 0.4
743	Typhoidin IIa (Nelson) 40 days	1:40	24	1.2 x 1.2, red area	1.3 x 1.3, R. I.	1.2 x 1.2, R. I.	1.9 x 1.9, red only	2.5 x 2.5, red
			48	N. P.	0.2 x 0.2	N. P.	0.5 x 0.5	1.2 x 1.2, red
744	Typhoidin Ic 40 days	1:100	24	1.8 x 1.7, R. I.	0.5 x 0.5, R. I.	1.1 x 1.1, R. I.	1.5 x 1.5, R. I.	0.8 x 0.8, R. I.
			48	1.3 x 1.3	0.6 x 0.6, R. I.	0.8 x 0.9, R. I.	0.5 x 0.5, R. I.	1.0 x 1.0, R. I.
757	Control		24	0.5 x 0.5, red spot	N. P.	N. P.	0.3 x 0.3	1.2 x 1.2, red
			48	0.4 x 0.4, red area	N. P.	N. P.	0.4 x 0.4, nodule	1 x 1, red
758	Control		24	N. P.	N. P.	N. P.	N. P.	N. P.
			48	N. P.	N. P.	N. P.	N. P.	N. P.

skin-reactions which may be considered specific. These findings therefore confirm those made by Gay and his co-workers⁴ on rabbits.

Observations made on rabbits immunized with bacteria other than typhoid, however, apparently show that the skin can react to various bacterial extracts. It is at once clear from the repeated occurrence of these reactions that they are nonspecific and cannot well be used as indices of immunity, for which this biologic test was primarily intended.

An explanation is best attempted on the basis that the typhoidin and similar reactions in rabbits are anaphylactic in nature and the result of an interaction of antigen and antibody. The observations of Gay and his co-workers,⁴ and our own experience, furnish evidence that such a conception is fully in harmony with the experimental evidence thus far collected. Typhoidin prepared with typhoid autolysates gives, in proper doses, skin reactions on immune or infected rabbits, which have every appearance of being specific. The complement-fixation tests with glycerin-extract preparations of the typhoid bacillus gave information with reference to the antigenic value of this typhoidin, and it was definitely established that protein substances, which gave complement-fixation only with a specific antiserum, were always present. Naturally, in these tests the typhoidin gave also group reactions with the serum of rabbits which had been immunized with closely allied organisms. As a rule, the extracts were suitable specific

antigens, even if they did not show as high an antigenic value as the pure typhoprotein. Probably dilution of the antigenic substances by the inactive precipitable polypeptids and other broth elements is in part responsible for this condition.

The suitable preparations are inactive on the skin of normal healthy rabbits, or they produce areolae which are not indurated and less than 1 cm. in diameter. On the other hand, in very small amounts, they produce intense reactions in immunized or infected rabbits. The intensity of the reaction runs parallel, in most of the tests, to the degree of immunization; the greater the concentration and number of injections of the bacterial suspension which has been inoculated, the stronger the reaction. The hypersensitiveness can be transferred to normal rabbits. Repeated intracutaneous tests produce a local cutaneous hypersensitiveness to the bacterial substances which are present in the extracts. In this connection it is of interest that such cutaneous hypersensitiveness can be present without appreciable increase or change of the serum antibodies.

Recent studies reported by Kolmer and his associates⁶ support these observations perfectly. He found in various infectious diseases of man and animal that cutaneous hypersensitiveness may exist without demonstrable antibodies in the body fluids.

The logical assumption from all of these facts is that cutaneous hypersensitiveness is the result of bacterial-protein sensitization. In the light of the conception of specificity it is difficult, however, to understand the many typhoidin reactions in rabbits treated with micro-organisms biologically and immunologically different from the typhoid bacillus. The nearest explanation would, in some instances, at least, be the primary toxic action of the glycerin extractives on the delicate rabbit skin. We feel that our preparations have been carefully tested on normal rabbits and usually found inert in those dilutions which caused reactions in the immune rabbits.

There is the possibility—analogueous to our findings in normal rabbits—that some of these rabbits possessed a marked individual hypersusceptibility to toxic albumoses of the extractives. It is, however, also evident from the tables that the immunized rabbits, which reacted apparently nonspecifically to bacterial proteins, showed no skin reaction with concentrated peptone solutions, which were always used in the control powders.

The fact that typhoid-immune rabbits react with paratyphoid, dysentery, and sometimes with other vegetable-protein extracts, and that rabbits immune to fowl typhoid, *B. bipolaris-septicus*, or those infected with tuberculosis, respond to typhoidin and similar products, suggests that the hypersensitiveness is the result of changes produced in the body of the animal by the process of immunization or infection.

In connection with the infections, additional information has recently come from the confirmatory tests of Dr. Traum, of the University of California, who was kind enough to carry out this observation on tubercular cattle and guinea-pigs. A nontoxic typhoidin in 10% solution produced, on intradermal injection into tubercular cattle, reactions which were similar in intensity to those obtained simultaneously with tuberculin. In the experience of Dr. Traum and ourselves, tubercular guinea-pigs also give reactions, which differ, however, from the true tuberculin reaction in size and induration.

The literature contains many similar observations that intercurrent diseases and local or general infections frequently cause cutaneous or general hypersensitiveness to various bacterial proteins. Mareck,¹⁵ for instance, found that horses suffering from pneumonia or scabies, would give positive ophthalmic or thermic reactions with typhin, a preparation similar to, if not identical with, our typhoidin. Südmersen¹⁶ observed typical local and general tuberculin and mallein reactions in horses which were free from these infections, but which had been inoculated with cultures of various bacteria for the preparation of antisera. Furthermore, Römer¹⁷ was able to kill tubercular guinea-pigs with a protein substance of the pneumobacillus of Friedlander and *B. pyocyaneus* which produced only a temperature reaction in healthy guinea-pigs. Petruschky¹⁸ observed in tubercular persons general hypersensitiveness to various bacterial products, and he even goes so far as to state that the hypersensitiveness to tuberculosis is due to a specific, and a nonspecific component. And again, luetin reactions have been produced in late syphilis by Boas and Ditlevsen¹⁹ with gonococcal and colon-bacillus suspensions. Several years ago one of us²⁰ reported, in confirmation of the observations of Bang²¹ and

¹⁵ Deutsch. tierärztl. Wchnschr., 1916, 24, p. 33.

¹⁶ Quoted by Mareck (see Reference 15).

¹⁷ Wien. klin. Wchnschr., 1891, 4, p. 835.

¹⁸ Ergebn. d. Inn. Med. u. Kinderheilk., 1912, 9, p. 557.

¹⁹ Arch. f. Dermat. u. Syph., 1913, 66, p. 852.

²⁰ Meyer, Jour. Med. Research, 1913, 29, p. 180.

²¹ Centrallbl. f. Bakteriöl., I, O., 1908, 51, p. 450.

Horné,²² that paratubercular cattle react locally and generally to an inoculation of avian tuberculin. This response certainly cannot be regarded as a group reaction of the avian tuberculo-protein with the anti-paratuberculo-protein, because the two organisms are only biologically related, as the tubercle bacillus is to the leprosy organism. Finally, Paiseau and Fixier²³ observed positive tuberculin reactions in typhoid patients, on the intracutaneous injection of this antigen, this reaction becoming negative, however, during convalescence.

Through the recent studies of Wells and Osborne²⁴ it is known that the specificity of an anaphylactic protein reaction is dependent on the chemical constitution of the reacting substances. One and the same protein molecules can, according to their conception, contain two or more specific substances which are capable of sensitizing the animal body. We were therefore not entirely unsupported when in previous paragraphs we expressed the opinion that the skin reactions noted with typhoidin in rabbits immunized against *B. bipolaris*, are due to sensitization with protein groups of identical chemical composition which, in all probability, are common to most of the bacteria—or even, perhaps, to the vegetable proteins in general. In the light of the observations of Wells and Osborne, it may be correct to assume that the complex typhoprotein contains parts of protein groups which are common also for the yeast or the *B.-bipolaris-septicus* protein. Extensive immunization or infection will finally give rise, aside from cutaneous hypersensitiveness, to the more prevalent specific typhoid proteins as well as to local or general hypersusceptibility to other protein fractions or complexes.

Furthermore, it has been shown by Wells and Osborne²⁵ that the antisera of rabbits which had received small doses of hordein gave complement-fixation only with this substance, but when larger doses were introduced, the reaction occurred also with gliadin, malt-proteoses, and so on. Such observations have also been made by us, and others, in connection with interagglutination tests in the paratyphoid group of bacteria.

It is possible that the same principle can be applied to the phenomena of anaphylaxis or sensitization in general; namely, that the degree of protein immunity has considerable influence on the speci-

²² Berl. tierärztl. Wchnschr., 1908, 23, p. 235.

²³ Compt. rend. Soc. de biol., 1909, 66, p. 877.

²⁴ Jour. Infect. Dis., 1913, 12, p. 341.

²⁵ Ibid., 1914, 14, 375.

ficity. An animal with a low immunity will only give reactions with the homologous, chemically (or biologically) analogous antigen; a highly immune or infected animal will also react with heterologous antigens which contain chemically closely related protein elements.

The nonspecific skin reactions observed in our experiments may be explained on this basis of sensitization. They are the result of a prolonged and intensive immunization with bacterial proteins which, considered as complexes, probably contain groups of protein elements found in all bacteria and perhaps in vegetables in general. The same mechanism may be at work in active disease and in infection. The specific cutaneous hypersensitiveness to homologous antigens, which is, as a rule, a fine indicator of general sensitiveness, apparently becomes, in a certain percentage of rabbits, gradually veiled during the course of immunization or disease by the development of nonspecific sensitization to heterologous antigens. The most intense nonspecific skin reactions were seen in animals which had been immunized with living organisms or had received more than 10 large intravenous injections.

It is necessary to mention here, however, that this form of sensitization does not occur with absolute regularity in every hyperimmune animal. And again, some observations indicate that over-immunization tends to reduce or wipe out cutaneous hypersensitiveness, just as it affects the serum antibodies. (This is particularly true for immunization with organisms of the *B.-dysenteriae* group.) How far individual factors and parasitic infections in rabbits influence the chances of nonspecific cutaneous hypersensitiveness, must still be investigated.

The explanation by Stokes¹⁰ of nonspecific skin reaction deserves further careful investigation on animals. We called attention to some atypical reactions the explanation of which by the assumption of a mechanism similar to that advanced by this writer, seems plausible. We failed to note in the rabbit the characteristic cycle of response on inoculation of colloids like agar, and therefore we lack experimental evidence to support the conception of Stokes.

The nonspecific reactions do not impair the diagnostic value of skin tests as a means of detecting hypersensitiveness to bacterial proteins, because as a rule the areola of the reaction to the specific antigen is larger and more intense than those of the reactions to the heterologous products. But they materially influence the conception that cutaneous hypersensitiveness can be used as an index of defensive activity of the part of the body fluids, or cells, against the bacterial

protein or against the living organism with which the skin reaction was obtained. How far our observations and explanations have a bearing on the diagnostic value of these tests in man, we are not at present prepared to say. Sufficient observations are published in the literature to show that, at least in tubercular persons, a nonspecific reaction with typhoidin may be expected in a certain percentage of cases.

CONCLUSIONS

Cutaneous hypersensitiveness of rabbits to typhoidin and similar preparations is, in all probability, the result of sensitization with typhoid or similar bacterial proteins. The hypersusceptibility to extract preparations of cultures can be passively transferred, and small amounts of typhoidin are capable of producing local sensitization of the skin to subsequent injections of the same, or similar, antigens.

In a fairly high percentage of hyperimmune or infected rabbits, nonspecific skin reactions follow the injection of extracts of pure bacterial proteins or microorganisms neither biochemically nor immunologically related to the organisms with which the animal has been treated. Some typhoid-immune rabbits show nonspecific reactions with paratyphoid and *B.-coli* extracts, which can be considered as group reactions; but frequently such animals, when highly immunized, will also give reactions with heterologous antigens not in the least related to *B. typhosus*. These reactions may be explained as the probable result of nonspecific sensitization with protein substances common to all bacteria or vegetables in general.

THE INTRACUTANEOUS 'TYPHOIDIN' REACTION*

III. THE RELATION OF CUTANEOUS HYPERSENSITIVENESS TO EXPERIMENTAL IMMUNITY AND INFECTION

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The conception that cutaneous hypersensitiveness is indicative of resistance to an infection, is not new. Basing his views on studies of tetanus and diphtheria toxin, von Behring¹ expressed the opinion that hypersusceptibility to toxin meant an increased defensive activity on the part of the body against the specific microorganisms. Moreover, extensive investigations by numerous workers, Römer,² Calmette,³ Krause,⁴ and others, on the relation of tuberculin hypersensitiveness to immunity in tuberculosis, demonstrated that sheep and also guinea-pigs protected against experimental infection with the tubercle bacillus possess, as a rule, a high cutaneous or general hypersensitiveness to tuberculin. Observations made by Römer⁵ as early as 1903 and, later, by one of us on a large number of bovovaccinated cattle in Pennsylvania demonstrated, however, apparent resistance to tuberculous infection without tuberculin hypersusceptibility. Furthermore, Krause⁴ maintained that sensitization of nontuberculous guinea-pigs with tubercle protein does not alter their resistance to experimental tuberculous infection. Marked tuberculin hypersensitiveness in man and cattle has, thus far, in the majority of cases been considered a sign of progressive tuberculous infection; if the disease subsides, the hypersensitiveness gradually falls to a low level.

In the light of our discussion of the nature of the typhoidin skin reaction and the conclusion reached that it probably indicates sensitization to some complex protein, it is quite evident that cutaneous hypersensitiveness cannot very well be accepted as an index of immunity.

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¹ Einführung i. d. Lehre v. d. Bekämpfung der Infektionskrankheiten, 1912, pp. 141, 353.

² Handbuch d. Technik u. Methodik der Immunitätsforschung, 1908, 1, p. 932.

³ Compt. rend. Soc. de biol., 1910, 68, p. 48.

⁴ Jour. Med. Research, 1911, 24, p. 399.

⁵ Handb. d. Technik u. Methodik der Immunitätsforschung, 1908, 1, p. 958, and Report of the Eighth International Veterinary Congress, Budapest, 1905, 1, p. 406.

It is possible that hypersensitiveness parallels the defensive activity of the body against the microorganism, but, as numerous observations have convinced us, absence of immunity certainly is not always associated with an absence of hypersensitiveness. Prolonged careful experiments are necessary to determine the interrelation of both conditions, and it is therefore with considerable hesitancy that we present in the following paragraphs some experiments which were mainly undertaken to show how difficult it is to conduct studies on typhoid immunity on rabbits. The technic of intravenous inoculation of many millions of typhoid organisms, which has to be chosen in order to produce conclusive results, is certainly a very drastic method of artificial infection, and is quite out of proportion to the natural mode of infection in man.

EXPERIMENT-GROUPS 1 AND 2

THE RELATION OF THE TYPHOIDIN TEST TO IMMUNITY

Two groups of experiments were carried out to determine the relation of the various skin reactions to the immunity of rabbits against subsequent infections by intravenous injections of large amounts of a recently isolated strain of *B. typhosus*, or by the injection of twice to three times the lethal dose of a fowl-typhoid culture. Most of the rabbits used in these groups were immunized with various types of vaccines, and were respectively tested with different kinds of typhoidin preparations.

Series 1.—Twelve rabbits (300 to 311 inclusive) had been immunized with 4 different kinds of vaccines. Rabbits 300, 301, 302, 309, 310, and 311 had each received 3 injections of an army type of vaccine; Rabbits 303, 304, and 305 had been treated with a sensitized—and Rabbits 306, 307, and 308 with a polyvalent typhoid-paratyphoid-A and -B vaccine. Every animal had received the amounts of vaccine ordinarily used for the prophylactic immunization of man, and at the customary intervals. The army and polyvalent types of vaccines had been given at 10-day intervals, and the sensitized vaccine on alternate days.

On November 1, or 34 and 46 days, respectively, after the last injection, the rabbits were tested with typhoidin and fowl-typhoidin preparations. These glycerin extracts had been used previously and found suitable in dosage of 0.001 gm. Our experience with this test is summarized in Table 1.

Immediately after this test, every rabbit was inoculated intravenously with one-thirtieth slant of a fowl-typhoid culture. The 6 rabbits which resisted this infection were tested again on March 15—105 days after the first test, or 139 and 151 days, respectively, after the last injection—with various typhoidin and *B. dysenteriae* extracts.

The preparations used had the following antigenic values: Typhoidin B, an extract of a 24-day-old glycerin potato-broth culture, contained 6.6 extract units (e.u.) per 0.001 gm. Typhoidin 3, the same preparation as was used in Group 1 (Table 3), had, at the time of test, only 33.3 e.u. per 0.001 gm. No

determinations of antigenic value were made on the previously tested glycerin Witte's-peptone-broth extract of *B. dysenteriae* Shiga (Do).

On April 13, 210 and 198 days, respectively, after the last injection of typhoid vaccine, or 162 days after the immunity test with fowl-typhoid bacilli, the 6 rabbits (301, 304, 307, 308, 309, and 311) were each inoculated intravenously with one-fourth slant of a 24-hour-old rabbit-blood agar culture of a recently isolated strain of *B. typhosus* (H-125). An interval of about 28 days after the typhoidin test was chosen to avoid any influence of the antigen on the antibody-production in the rabbits to be tested. Five normal rabbits (641 to 645) served as controls.

This extensive experiment is summarized in Table 1. In the first typhoidin tests there were positive reactions in all the vaccinated rabbits, with the exception of 306 and 307, which had been injected with a trivalent typhoid-paratyphoid vaccine. All the skin reactions were distinct, but fluctuations in the size of the areola and the degree of induration were noticed among the rabbits of one and the same lot. Vaccines A and B provoked the best reactions; those to Vaccine D were fair in some animals, the areola being not much larger than in the control rabbits. No parallelism between intensity of reaction and amount of serum agglutinins could be demonstrated. Of interest, however, was the striking parallelism exhibited in the degree of skin reaction of the same rabbit (see 300, 302, 303, 304, etc.) to fowl typhoidin. Again, in 306 and 307 reactions were absent, and in 305 and 308 they were marked and persisted well up to the 24th hour. The areolae were found to be very slight or indistinct in the rabbits which had failed to respond distinctly to typhoidin (310 and 311). Unfortunately, at the time these tests were carried out we did not consider it necessary to make 48-hour readings, but it will be noticed in Table 1 that the typical reactions either were the same at the 24-hour reading, or had increased in intensity over those of the 16th hour. It may be assumed, therefore, that the reactions would have persisted up to the 48th hour. In the amount of 1 mg. chosen for these tests, typhoidin and fowl-typhoidin extracts were only slightly irritant to the skin of normal rabbits.

The immunity tests of the rabbits protected with Vaccine A showed that the 2 rabbits (300 and 302) which gave the best skin reactions, succumbed to infection with fowl-typhoid organisms. In the group of rabbits injected with Vaccine B., one animal died from the infection, while another (305) had become so nonresistant as a result of the intoxication that it fell an easy prey to a secondary infection with *B. cuniculisepticus*. Both animals had given good positive skin reactions which differed slightly from those of Rabbit 304, which resisted

RESULTS OF TESTS IN EXPERIMENT-GROUP 1

W. D. = well-defined areola
R. I. = deep purplish, indurated area
I. R. = indurated red area

= very slight induration

S. O. = slight edema

D. R. I. = diffusely indurated

S. spot = small spot

A. P. = needle puncture, traumatic reaction

THIMBLE RABBITS. Five, or 83.3%, failed because entirely or succumbed to acute infection

TABLE 2
RESULTS OF TESTS IN EXPERIMENT GROUP 2

Rab- bit	Immuniza- tion	Last Injec- tion	Pre- vious Test	Serologic Test March 25		Hours	Typho- id 16 1:50	Typho- id 16 1:100	Control Powder 1:10	Paraty- phoid B 1:50	B. Coll 16 1:50	Tubere- culi 1:50	Hours	Fowl-Ty- phoid 16 1:50	Fowl-Ty- phoid 16 1:100	Control Powder	Staphylo- cocci 1:50	Paraty- phoid-B 1:50	Dysentery Do. 1:50	Immunity Test for Fowl Typhoid	Result	Immunity Test for Typhoid	Result
				Agglutina- tion	Com- plement Fixation																		
340	A. vaccine 3 injections	Nov. 30	Jan. 23	1:400	0.05 A* 0.005 S	16	1.9 x 1.9	1.7 x 1.7	Spot	2 x 2	2.9 x 2.9	Spot	16	2.4 x 2.4	1.8 x 1.8	N. P.	2.5 x 2.5, I. R.	1.2 x 1.2	1.4 x 1.4, R. I.	April 18, 1/10 slant of fowl-ty- phoid bacilli (3 times lethal dose)	Died from fowl typhoid April 21		
						24	2.7 x 2.3	1.5 x 1.5	2.4 x 2.4	2.4 x 2.7	2.7 x 2.7	2.1 x 2.1	24	2.2 x 2.2	1.4 x 1.4	Red spot	2.5 x 2.5	1.5 x 1.5	1.6 x 1.6				
						48	2.5 x 2.5	2 x 2	2.2 x 2.2	2.2 x 2.7	2.3 x 2.3	2.1 x 2.1	48	2.1 x 2.1	1.4 x 1.4	N. P.	2.2 x 2.2	0.6 x 0.6	1.3 x 1.3				
341	A. vaccine 3 injections	Nov. 30	Jan. 23	1:600 —	0.05 A	16	2.3 x 2.3, R. I.	Diffuse red area	Spot	2.9 x 2.9, R. I.	2.1 x 2.1, Diff. and Ind.	Red spot											
				1:1000 —	0.05 S	24	2.4 x 2.4	1.4 x 1.4	Spot	2.1 x 2.1, R. I.	2.5 x 2.5, Diff. and Ind.	Red spot											
						48	2.5 x 2.5	1.4 x 1.4	1.4 x 1.4	2.4 x 2.4, R. I.	2.9 x 2.9, R. I.	1.3 x 1.3											
						72	1.7 x 1.7	N. P.	Papule	N. P.	2.5 x 2.5, R. I.	Red spot											
342	B. vaccine 3 injections	Nov. 16	Jan. 23	1:100 —	0.05 A 0.05 S	16	1.9 x 1.9, R. I.	1.3 x 1.3	Spot	1.6 x 1.6, R. I.	1.3 x 1.3, R. I.	Spot	16	2.5 x 2.5	1.6 x 1.6	N. P.	N. P.	0.2 x 0.2	N. P.	April 18, 1/10 slant of fowl-ty- phoid bacilli	Died from fowl typhoid April 20		
						24	1.6 x 1.6, R. I.	1.3 x 1.3	Spot	1.3 x 1.3, R. I.	1.3 x 1.3	Spot	24	1.9 x 1.9	1.6 x 1.6	N. P.	N. P.	1.4 x 1.4	N. P.				
						48	1.7 x 1.7, R. I.	0.9 x 0.9	N. P.	1.1 x 1.1	1.5 x 1.5	Spot	48	1.5 x 1.5	1.3 x 1.3	N. P.	Red spot	Red spot	N. P.				
						72	Spot	Spot	Red spot	Red spot	1.4 x 1.4, red area	Spot											
343	B. vaccine 3 injections	Nov. 16	Jan. 23	1:200 —	0.05 A 0.05 S	16	2.1 x 2.1	1.7 x 1.7	Red spot	1.8 x 1.8, I. R.	1.9 x 1.9, R. I.	1.8 x 1.8, R. I.											
						24	2.4 x 2.4	1.9 x 1.9	Red spot	2.3 x 2.3, R. I.	1.8 x 1.8, R. I.	1.6 x 1.6, red											
						48	2.3 x 2.3	1.7 x 1.7	N. P.	2.1 x 2.1, R. I.	2 x 2, red	1 x 1, red											
						72	Red spot	Red spot	N. P.	N. P.	2 x 2, red area	N. P.											
344	B. vaccine 3 injections	Nov. 16	Jan. 23	1:200 —	0.05 A 0.05 S	16	2 x 2	1.7 x 1.7	N. P.	N. P.	1.3 x 1.3, S. I.	Red spot											
						24	2.6 x 2.6	1.9 x 1.9	N. P.	Red spot	Diffuse area	Red spot											
						48	2.5 x 2.5	1.6 x 1.6	N. P.	1.4 x 1.4	1.4 x 1.4	1.5 x 1.5											
						72	Red spot	Red spot	N. P.	N. P.	2 x 2, red area	N. P.											
346	C. vaccine (polyval- ent) 3 injections	Nov. 30	Jan. 23	1:300 —	0.05 A <0.05 S	16	1.6 x 1.6, W. D.	1.1 x 1.1, R. I., good	Red spot	2.1 x 2.1, R. I.	1.5 x 1.5, R. I.	1 x 1, R. I.	16	2.4 x 2.4, Diffuse	1.6 x 1.6, Diffuse	Red spot	N. P.	N. P.	Red spot	Red spot	April 18, 1/10 slant of fowl-ty- phoid bacilli	Died from fowl typhoid April 21	
						24	1.7 x 1.7, R. I.	0.9 x 0.9	Spot	1.9 x 1.9, R. I.	1.5 x 1.5, R. I.	1 x 1, R. I.	24	1.8 x 2.6	1.7 x 1.7	N. P.	N. P.	1.6 x 1.6	1.5 x 1.5				
						48	1.6 x 1.6	0.9 x 0.9	0.4 x 0.4	1.6 x 1.8, R. I.	2 x 2, R. I.	0.8 x 0.8, R. I.	48	2.2 x 2	1.5 x 1.5	N. P.	N. P.	1.4 x 1.4	2.1 x 2.1				
						72	Red spot	N. P.	N. P.	Red spot	2 x 2, R. I.	N. P.											
347	C. vaccine (polyval- ent) 3 injections	Nov. 30	Jan. 23	1:600 —	0.05 A 0.05 S	16	2.1 x 2.1, W. D.	1.7 x 1.7	Spot	2.1 x 2.1, R. I.	2.4 x 2.4, R. I.	1.6 x 1.6, R. I.											
						24	2.0 x 2.4, W. D.	1.2 x 1.2	Spot	1.5 x 1.5, R. I.	2.1 x 2.1, R. I.	1.4 x 1.4, R. I.											
						48	2.4 x 2.4	0.5 x 0.5	Spot	2.1 x 2.1, R. I.	2.7 x 2.7, R. I.	0.9 x 0.9, R. I.											
						72	Red area	Red spot	N. P.	N. P.	2.5 x 2.5, R. I.	N. P.											
348	D. vaccine 3 injections	Nov. 30	Jan. 23	1:300 —	0.05 A 0.05 S	16	1.5 x 1.6, R. I.	Red spot	Red spot	1.5 x 1.5, D. R.	1.8 x 1.8, R. I.	1.6 x 1.6, R. I.											
						24	1.8 x 1.8	N. P.	N. P.	1.9 x 1.9	1.9 x 1.9	1.7 x 1.7											
						48	2.1 x 2.1, R. I.	N. P.	N. P.	2.5 x 2.5, R. I.	1.8 x 1.8, red area	0.9 x 0.9											
						72	Red spot	N. P.	N. P.	Red spot	Red Ind.	N. P.											
349	D. vaccine 3 injections	Nov. 30	Jan. 23	1:100 —	0.05 A 0.05 S	16	Red spot	Diffuse red spot	N. P.	1.9 x 1.9, D. I.	Diffuse area	Diffuse area	16	2.2 x 2.2	1.8 x 1.8	N. P.	N. P.	N. P.	0.5 x 0.5, R. I.	April 18, 1/10 slant of fowl-ty- phoid bacilli	Died from fowl typhoid April 20		
						24	1.9 x 1.9	1.0 x 1.0	N. P.	2.1 x 2.1, R. I.	3.1 x 3.1, R. I.	0.9 x 0.9, red spot	24	1.8 x 1.8	1.6 x 1.6	N. P.	N. P.	N. P.	1.5 x 1.5, R. I.				
						48	1.6 x 1.6	N. P.	N. P.	Red spot	Red area	N. P.	48	1.7 x 1.7	1.2 x 1.2	N. P.	N. P.	N. P.	1 x 1, diff. swelling				
350	D. vaccine 3 injections	Nov. 30	Jan. 23											
352	Control	Negative	0.1 (50%)	16	N. P.	0.6, cond. Indur.	0.5, S. I. Spot	Red spot	0.5, S. I. spot	N. P.											
						24	N. P.	0.2, cond. Indur.	N. P.	Red spot	0.5, S. I. spot	N. P.											
						48	N. P.	N. P.	N. P.	N. P.	2.2 x 1.1, red	N. P.											
						72	N. P.	N. P.	N. P.	1 x 1	2.2 x 2.2, R. I.	N. P.											
353	Control											
356	Control 1/10 slant of fowl typhoid											

* A = antitoxin titration. S = serum titration.

† Controls in immunity test, 350, 352, 353, 356, were inoculated at the same time and examined postmortem 1 month later. One rabbit (352), or 35%, died from typhoid septicaemia. Immune rabbits: 3, or 66%, died or became carriers.

the infection. In Vaccine-group C the results are even more contradictory to the conception that cutaneous hypersensitiveness is indicative of immunity; 1 rabbit (306) with a negative skin reaction died acutely from the infection, while the mate (307), which showed very little sensitization, together with an animal (308) which reacted positively, resisted the infection.

Similar conditions developed in Vaccine-lot D, in which a non-reacting rabbit overcame the immediate effect of the fowl-typhoid bacillus, but died later of a secondary infection. Of the 2 rabbits which resisted the infection, 1 gave a slight reaction with fowl typhoidin (311). These observations are in harmony with the work of Theobald Smith and Ten Bröeck,^a who have presented evidence that the human-typhoid bacillus tends to protect animals against the toxins of the fowl-typhoid bacillus, a finding which we were able to confirm in previous experiments. It was for this reason that we chose the fowl-typhoid bacillus as a test organism.

In comparison with the control rabbits (469, 470, 471, and 537), all of which succumbed to the intoxication and infection in from 2½ to 3 days, the typhoid-vaccinated rabbits showed a certain degree of resistance to the infection. The heart-blood cultures contained few or no organisms, while those of the controls were strongly positive.

The resistance of some of the rabbits to fowl-typhoid bacilli was not indicated by exceptionally marked skin reactions. Rabbits with small and doubtful skin reactions showed as much resistance as those which had extensive cutaneous areolae. It is therefore impossible to conclude from these tests that any relationship exists between the immunity of an animal to an overwhelming infection with the fowl-typhoid bacillus and the presence, or absence, or intensity, of a skin reaction produced by the intracutaneous inoculation of a glycerin extract of this particular test organism.

The remaining 6, apparently healthy, rabbits, when tested several months later with 2 types of typhoidin, responded well with 1 preparation. Only 1 rabbit (301) gave, with 0.00025 gm. of typhoidin B, an areola lasting longer than 24 hours. The positive reactions with Typhoidin 3 were well defined and lasted for at least 48 hours; some of the reactions were very intense and were indicated by diffuse edema, which often made it impossible to read the diameter of the areola. In the dosage of 0.00025 gm. chosen, the dysentery extract was inactive.

^a Jour. Med. Research, 1914-1915, 31, p. 545.

The sera of most of the rabbits had low agglutinin and complement-fixing antibody content; only 1 rabbit (308) showed an agglutination of from 1:1000 to 1:4000. On subsequent immunity tests, Rabbits 307 and 308 died acutely from typhoid intoxications, Rabbits 301, 304, and 311 became carriers (gall-bladder), and only 1, Rabbit 309, resisted the infection.

If the skin reactions of these animals are considered as indices of immunity, it is well to record that Rabbit 309, in the first 24 hours, gave, so far as we could discern, a better reaction than all of the others, yet the skin of this rabbit reacted also to the control powder 3 c. Therefore, it is not unlikely that this rabbit possessed a hypersensitive-ness, individually, more marked than in the other rabbits. That this single positive result has little value is further shown by a comparison of the other rabbits. It is also clear from Table 1 that all the rabbits which gave similarly strong reactions on the first and second tests either became carriers, or, as in the case of Rabbits 307 and 308, did not even resist the acute intoxication. In this connection it may be well to call attention to the fact that of 5 normal rabbits (641 to 645) which served as controls for the immunity tests, not one succumbed to the intoxication, and only 3, or 60%, became carriers. Five immune rabbits, or 83%, became carriers or succumbed to the infection.

We are unable to produce evidence that a positive skin test permits of the conclusion that a rabbit will resist a subsequent injection of a large dose of living typhoid bacilli (6000 millions). Data collected from other experiments which will be published in the near future, indicate that the typhoid-carrier stage in rabbits is strongly influenced by the existing serum immune bodies of the animal, and by other factors — biliary passages, bile, etc. — which have probably nothing to do with the cutaneous hypersensitiveness to typho- or bacterial proteins.

Series 2.—Ten rabbits (340, 341, 342, 343, 344, 346, 347, 348, 349, 350) were immunized with the same types of vaccines as were used in Series 1. Only 2 rabbits (340 and 341) were inoculated with Vaccine A; 3 (342, 343, and 344) with Vaccine B; 2 (346 and 347) with Vaccine C; and 3 (348, 349, and 350) with Vaccine D.

On January 28, 59 and 73 days, respectively, after the last injection, these rabbits were tested with Typhoidin 3 and Fowl-typhoidin 3. The results of this test have been discussed in Group 1 (Table 3, 1st paper).

On March 30, 121 days and 135 days, respectively, after the last injection, the animals were tested again with various typhoidin, paratyphoidin-B, tuberculin, and B.-coli extracts; on April 10, 1 rabbit of each lot was also tested a

second time with fowl-typhoidin, paratyphoidin-B, staphylococcus, and dysentery (Do) extracts.

The glycerin extracts used—with the exception of the tuberculin—were 1-month-old potato-broth cultures, concentrated and precipitated as usual. Typhoidin 16 had an antigenic value of 5 e.u. per milligram. No determinations were made for the paratyphoidin-B and B.-coli extracts. The fowl-typhoidin 4 and paratyphoidin B have been described in Group 2 (Table 3, 2nd paper).

These rabbits were infected intravenously with fowl typhoid bacilli on April 18, 139 and 153 days, respectively, after the last injection. The remaining 5 rabbits (341, 343, 344, 347, 348) were tested as usual with one-half slant of a rabbit-blood agar culture of a recently isolated strain of *B. typhosus*.

The results of these tests are shown in detail in Table 2. With typhoidin and Fowl-typhoidin 3, positive skin reactions were obtained in every rabbit. The degree and the persistence of the reaction, up to or after the 48th hour, were somewhat better in the A and C than in the B and D vaccine lots. That typhoidin in quantities smaller than 1 mg. calls forth a better response, was quite evident in the few tests in which each rabbit of the respective lots was tested with dilutions of the antigen. This observation confirms the findings of Force⁷ that an army type of vaccine gives, in man, better sensitization than some of the other vaccine preparations.

The degree of the reaction apparently does not correspond with the amount of serum immune bodies demonstrable in the form of agglutinins and complement-fixing antibodies. This absence of parallelism is more pronounced in the second test, in which a low serum immunity is present together with a very marked and intense reaction. Every rabbit reacted to 0.02 gm. of Typhoidin 16, some of the reactions being strong and remaining so for 48 hours and longer. Several rabbits responded to the second typhoidin test as they did to the first. For example, in 342, 346, and 349 the areolae were less indurated and smaller, and faded more rapidly, than in the other rabbits. With 0.001 gm. of typhoidin, Rabbit 348 gave a negative reaction, and 342 and 346 gave very slight reactions.

These observations might be construed as having some significance, because neither animal resisted a subsequent fowl-typhoid infection, and the serum immune bodies were, on the average, low. One must recall, however, that these rabbits represent 3 different lots of vaccines of various composition, and that no definite conclusions can be drawn from so few observations.

The other glycerin extract chosen in this experiment, in dilution of 1:50, produced extensive nonspecific reactions. This was particularly

⁷ Personal communication.

true of the B.-coli extract. Small nodules the size of peas, with small centers containing pus, persisted for from 4 to 6 days.

In our opinion these reactions were due to the leukotactic influence of the bacterial proteins, which were more concentrated in the B.-coli powder than in the other preparations on account of the heavier culture from which it was precipitated. The paratyphoidin-B extract gave skin reactions of varying degrees of intensity, but must be considered unreliable for the same reason.

The tuberculin preparation, prepared from a bovine strain, was filtered before being precipitated, and tested on numerous normal rabbits, with negative results. Rabbits 346, 347, and 348 gave skin reactions which, in regard to the diameter of the areolae and persistence of hyperemia and induration, must be regarded as suspicious. Several animals (343, 344, etc.) apparently showed a slight degree of cutaneous hypersensitiveness to a concentrated tuberculin, by small slightly indurated areolae. Two rabbits (342 and 349) which throughout the tests gave negative or doubtful typhoidin reactions, failed to develop skin reactions with tuberculin. The tuberculin reactions are the result of nonspecific cutaneous hypersensitiveness developing as a consequence of local sensitization with various extracts. This phase of the problem has been discussed in the previous paper.

The 4 rabbits (340, 342, 346, 348) which were tested with fowl typhoidin 10 days after the last general test, all reacted positively, the areolae being well-defined for 48 hours. The response to other glycerin extracts confirmed previous observations; that is, 342 failed to respond to the dysentery extract, which caused slight skin reactions in the remaining 3 rabbits. With the exception of 340, these rabbits all reacted to the staphylococcus extract. This particular animal was, apparently, hypersensitive to certain elements of the broth, for on previous tests it had reacted distinctly with the control powders. The result of this test is of very little value in the light of our subsequent findings on acquired local hypersensitiveness.

The 4 rabbits (340, 342, 346, 348) which reacted positively to fowl typhoidin in the first test (Table 3, 1st paper) and in the subsequent tests just discussed, did not resist an injection of 3 times the lethal dose of the fowl-typhoid bacillus. Until further experiments have been carried out, it is difficult for us to decide whether these animals died of the intoxication or of the infection. In the light of the state-

ments of Theobald Smith⁶ and Bull,⁸ the protection against fowl typhoid and typhoid, respectively, obtained by immunization with dead or living organisms is also directed against the toxins. We mention these points here because in recent studies on typhoid immunization the view has been expressed that the resistance against intoxication and that against infection are probably two separate functions in the defensive activity of the body. We are not as yet prepared to commit ourselves on this point, but record at this time some observations which show clearly that typhoid-immune rabbits are capable, to an extent, of handling the infection in the blood stream; for, at the time of death, the heart and portal blood of the 4 rabbits contained very few organisms (from 3 to 4 colonies per drop of blood), while the liver, spleen, and particularly the bile, contained a very large number of fowl-typhoid bacilli. In the control rabbits (655 and 656) just the reverse was the case; the blood was rich in bacteria, while the bile and organs contained comparatively few or no microorganisms.

The result of this immunity test with fowl-typhoid bacilli confirms the observations of Series 1 and supports the contention that a positive allergic skin reaction in a rabbit is not always proof that this type of animal is resistant to an infection or an intoxication with the organisms used as an antigen in the skin test.

Of the remaining 5 rabbits which represent the 4 types of vaccines, only 2 resisted an intravenous injection of a large dose of living typhoid organisms. Two animals became carriers, and 1 died suddenly of intoxication. The latter animal had developed a fair amount of serum immune bodies following the immunization, and had always responded well with a marked skin reaction. The same may be said of the other two rabbits, 347 and 348; they differed very little with regard to immune bodies and skin reactions from Rabbits 341 and 344, which resisted the infection. Rabbit 341 possessed more agglutinins and gave in the first typhoidin test a somewhat better reaction, but the differences were so slight that they are not worth further consideration (compare Table 3, 1st paper).

In comparison with the 4 control animals (632, 640, 669, 700), of which only 1, or 25%, succumbed to the infection, 3, or 60%, of the immune rabbits died or became carriers (gallbladder).

The conclusions derived from Series 1 are therefore supported by Series 2. Until we know more about the mechanism governing the

⁸ Jour. Exper. Med., 1916, 24, p. 35.

typhoid-carrier stage and the immunity of the rabbit to typhoid, the only conclusion justifiable is that a positive allergic skin reaction in a rabbit does not indicate that this animal will resist a subsequent intravenous injection of living typhoid bacilli, or that the rabbit is so protected that it will not become a chronic carrier of bacilli in the gall-bladder or liver.

These experiments suggest, furthermore, that in rabbits cutaneous hypersensitiveness to various kinds of bacterial proteins may exist without the least degree of effectual resistance to the corresponding microorganism.

In the tables given in the preceding papers, we have presented the results of some of the immunity tests under the heading "Remarks." Whenever possible, each rabbit was tested after a certain time interval with the same strain which had been used for the immunization. From one-tenth to one-half agar slant was inoculated intravenously, and the result was determined by autopsies in from 1 week to 1 month, or even longer. For example, in Table 3 of our 1st paper, the outcome of the immunity tests in Rabbits 319, 325, 326, and 427, and so on, supports our views that a strongly positive skin reaction does not indicate that a particular animal is protected against an experimental infection. In the present communication it is impossible to call attention in detail to all of these interesting results, but we feel that the evidence collected corroborates fully the facts demonstrated in Experiment-group 1.

RELATION OF THE CUTANEOUS REACTION TO THE SERUM IMMUNE BODIES

It is not our intention to enter into a discussion of the relative values of the serum immune bodies as indices of immunity. The various tests for agglutinins and complement-fixing antibodies are so simply carried out that it is well to choose them as guides during the process of immunization and to consider them as an index of a response as well as presumptive evidence of the presence of other antibodies. The recent studies of Bull⁹ on intravital agglutination contributed important information relative to the value of agglutinins, and in connection with the typhoid-carrier problem in rabbits we have found that these immune bodies may play an interesting rôle.

⁹ Jour. Exper. Med., 1915, 22, p. 475.

In analyzing the various serum findings reported in the different tables (also 1st and 2nd papers) we fail to find any parallelism between the agglutination titer, the amount of complement-fixing antibodies, and the intensity of the skin tests. Rabbits with a high agglutination titer frequently gave less pronounced cutaneous reactions than those with a low titer, and vice versa. In addition to this fact it was quite apparent that rabbits which were immunized against *B. bipolaris* produced an antiserum free from agglutinins or complement-fixing antibodies for *B. typhosus*, and yet showed intense cutaneous hypersensitiveness to this particular antigen. And again, rabbits which had lost their serum immune bodies would still give anaphylactic skin reactions.

Drawing conclusions from our observations on rabbits, we may state that no definite relationship exists between agglutinins and complement-fixing antibodies and cutaneous hypersensitiveness to typhoidin or similar glycerin extracts. We believe, furthermore, that sensitization to typhoidin persists for a longer period in rabbits than do serum antibodies, and that hypersensitiveness may exist even in the absence of demonstrable immune bodies.

EXPERIMENT-GROUP 3

RELATION OF THE SKIN REACTION TO THE CARRIER STATE

Cutaneous tests with tuberculin, sporotrichosin, streptothricin, etc., have thus far proved to be of inestimable value as a means of diagnosing an existing tubercular, sporotrichotic, or streptotrichotic infection (Claypole¹⁰). It is apparent from these facts that allergic tests have, thus far, only been employed successfully in chronic diseases, and their value in acute infectious diseases is, as previously stated, as yet undecided. Until very recently a positive skin reaction was considered an index of an existing infection and rarely as one of immunity. Römer¹¹ even went so far as to state that the intensity of the cutaneous reactions of a guinea-pig to tuberculin depends directly on the extent and advance of the tuberculous process. Krause¹² supported and enhanced these statements by additional observations, which we will consider later. The same conclusions were reached by us in some experiments with sporotrichosin on sporotrichotic rats, rabbits, and 1 human case.

¹⁰ Arch. Int. Med., 1914, 14, p. 104.

¹¹ Beitr. z. Klin. f. Tuberk., 1909, 14, p. 1.

¹² Jour. Med. Research, 1916, 35, pp. 1, 21.

TABLE 3
RESULTS OF TESTS IN EXPERIMENT-GROUP 3
DATE OF TESTS: APRIL 10, 1916

Rabbit	Immunization	Result of Previous Test	Date of Reinjection (1916)	Serologic Tests April 10, 1916		Hours
				Agglutination	Complement-Fixation	
317	B. typhosus (Rawlings)	Feb. 3 See Table 3, Paper 1	Jan. 25	1:2000		16 24 48
332	B. typhosus 46	Feb. 3 See Table 3, Paper 1	March 2	1:2000 (1:800 Feb. 22)	0.003	16 24 48 72
335	B. typhosus 49	Feb. 23 See Table 6, Paper 1	March 2	1:10000 (1:600 Feb. 22)	A 0.05 * S 0.005	16 24 48 72
336	B. typhosus 50	Feb. 3 See Table 6, Paper 1	March 2	1:6000 to 1:8000 (1:2000 to 4000) (Feb. 22)	>0.003	16 24 48 72
337	B. typhosus 48	Feb. 23 See Table 5, Paper 1	March 2	1:4000 (1:4000 Jan. 14)	0.0005	16 24 48 72
353	B. typhosus 70		3 injections previous to Nov. 13, 1915	1:600 (1:6000 Dec. 28)	>0.003	16 24 48 72
355	B. typhosus 73	Feb. 23 See Table 6, Paper 1	March 3	(1:4000 Mar. 11) (1:1000 Feb. 22)	0.003	16 24 48 72
358	B. typhosus 78	Feb. 23 See Table 5, Paper 1	March 3	1:6000 (1:800 Feb. 22)	A 0.02 * S 0.0005	16 24 48 72
359	B. typhosus 79		March 3	1:6000 (1:800 Feb. 22)	A 0.02 * S 0.0005	Died during the test
261	B. typhosus 94	Feb. 23 See Table 6, Paper 1	March 3	1:6000 (1:4000 Feb. 21)	A 0.02 * S 0.0003	16 24 48 48
607 and 608	Controls repeatedly tested within 1½ months					
638	Control			1:10	0.05	16 24 48 72
639	Control			0	0.1 (50%)	16 24 48 72
638	Control			0	0.1 (50%)	16 24 48 72

A = antigen titration.

S = serum titration.

TABLE 3—Continued

RESULTS OF TESTS IN EXPERIMENT-GROUP 3

DATE OF TESTS: APRIL 10, 1916

Typhoidin P. C.		Paratyphoidin A 1:100	B. Coli 1:100	B. Paratyphoidin B 1:100	Control Powder	Remarks
1:50	1:100					
(cm.) 2.2 x 2.2 1.8 x 1.8 1.9 x 1.9	(cm.) 1.2 x 1.2 1.4 x 1.4 1.4 x 1.4	(cm.) 1.1 x 1.1 1.2 x 1.2 Red spot	(cm.) Red spot Red spot 1.8 x 1.8, delay	(cm.) Red spot Red spot N. P.	(cm.) N. P. Red spot Red spot	Not a carrier
1.8 x 1.8 1.9 x 1.8 1 x 1 N. P.	1.6 x 1.6 D. O. 0.8 x 0.8 N. P.	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	N. P. N. P. 0.8 x 0.8 N. P.	Red spot Red spot N. P. N. P.	Not a carrier
2.4 x 2.4, W. D. 3 x 3 W. D. 2.2 x 2.2, W. D. N. P.	1.2 x 1.2, W. D. 1.8 x 1.8, W. D. 1.3 x 1.3, W. D. N. P.	Red area 1 x 1 Red spot N. P.	Red area 1 x 1 Red spot N. P.	0.3 x 0.3 Red spot Red spot N. P.	Red spot Red spot N. P. N. P.	April 19, 1916, B. typhosus in liver and gall-bladder wall
2.4 x 2.4, W. D. 3 x 3, W. D. 2.3 x 2.3, W. D. Red area	2 x 2, W. D. 1.7 x 1.7, W. D. 1.6 x 1.6, W. D. Red spot	1.5 x 1.5, R. I. 1 x 1 1.2 x 1.2 N. P.	Diffuse red area 1.8 x 1.8 1.3 x 1.3 Red area	Diffuse red area N. P. N. P. N. P.	N. P. Red spot Red spot N. P.	April 10, 1916, B. typhosus in bile and gall-bladder wall
1.6 x 1.6 1.9 x 1.9 1.1 x 1.1 N. P.	1.4 x 1.4 1.6 x 1.6 N. P. N. P.	1.1 x 1.1 1.1 x 1.1 N. P. N. P.	N. P. 2.2 x 2.2 2.3 x 2.3 N. P.	Red spot 1.1 x 1.1 N. P. N. P.	N. P. N. P. N. P. N. P.	April 19, 1916, not a carrier
N. P. Red spot 1.3 x 1.3 Red spot	Red spot Red spot Red spot N. P.	Red spot Red spot N. P. N. P.	Red spot Diffuse red area 2 x 2 N. P.	Red spot Red spot N. P. N. P.	Red spot Red spot N. P. N. P.	Did not become carrier; bile sterile
Red area 2.4 x 2.4, W. D. 0.8 x 0.8, R. I. Red spot	Diffuse red area 1.8 x 1.8, W. D. Red spot N. P.	Red spot Red spot N. P. N. P.	Red spot Red spot N. P. N. P.	Red spot Red spot N. P. N. P.	Red spot Red spot N. P. N. P.	April 22, 1916, B. typhosus in gallbladder wall
1 x 1 1.5 x 1.5, W. D., very good 0.8 x 0.8 Small nodule	1.2 x 1.6 1.5 x 1.5, W. D. 0.8 x 0.8 Red spot	0.6 x 0.6 0.8 x 0.8 N. P. N. P.	1.4 x 1.4 1.4 x 1.4 1 x 1 Red spot	1.8 x 1.8 1.1 x 1.1 Nodule Red spot	Red spot Red spot N. P. N. P.	Emaciated and clinically sick; B. typhosus in bile
						April 11, 1916, B. typhosus in bile and gall-bladder wall
2.2 x 2.2, W. D. 2.4 x 2.4, W. D. 1.8 x 1.8, W. D. Red nodule	2.1 x 2.1, W. D. 2 x 2, W. D. 1.3 x 1.3, W. D. Red spot	1.2 x 1.2 1.1 x 1.1 Red spot N. P.	1.5 x 1.5 2 x 2 1.8 x 1.8 Red area	1.5 x 1.5 1.3 x 1.3 1.8 x 1.8 Red spot	1.5 x 1.5 Red area 1 x 1 Red area	Emaciated and clinically sick. April 14, 1916, B. typhosus in bile
N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	
N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	N. P. St. swelling 0.5 x 0.5 Red spot	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	
N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	Red spot 0.8 x 0.8 0.1 x 0.4 Red spot	N. P. N. P. N. P. N. P.	N. P. N. P. N. P. N. P.	

The scope of our investigation therefore called for a study of cutaneous hypersensitiveness in rabbits which harbored the typhoid bacilli somewhere in the body. From previous experiments we had derived practice in producing 'gallbladder' carriers by the intravenous injection of large doses of living typhoid germs into immune rabbits. For numerous reasons it was impossible to ascertain with certainty when the animals began to harbor the organisms in the liver or gallbladder (stool cultures, even in good carriers, being often negative), until after the skin test had been completed. The striking results which we obtained in Experiment-group 3 were therefore observed at a time when we had no knowledge of the degree of typhoid infection.

Nine rabbits (332, 335, 336, 337, 355, 358, 359, 387, 361) possessing a basic immunity, had been inoculated intravenously on January 25, March 2, and March 3, respectively, with one-half rabbit-blood agar slant of the same typhoid strain which had been used for immunization. Previous to this test inoculation on Feb. 2 or 23, most of these rabbits had received an intracutaneous typhoidin test. The results of this test are shown in Tables 3 and 5 of our 1st paper. One rabbit (353) which had thus far received only 3 small injections of a killed typhoid culture previous to Nov. 13, 1915, together with 3 normal rabbits (638, 639, and 698) and 2 rabbits (607 and 608) which had been tested intracutaneously on a previous occasion, served as controls.

Most of these rabbits were killed and examined in from 2 to 16 days after the test, and it was found that Rabbits 335, 336, 355, 358, 359, and 361 were carriers of the bacilli in gallbladder or liver, and therefore were infected at the time of the typhoidin test. Three rabbits (358, 359, and 361) were considerably emaciated and anemic at the time of the test, one (359) even died during the experiment. It was very fortunate that we incorporated in the experiment some rabbits which had undergone the same treatment, but which had not developed a carrier condition. The immune bodies were present in the animals in similar or even larger amounts than in the carriers, and these were therefore most suitable for work on the problem of this experiment.

The test preparation used in these instances was a typhoidin obtained from a 41-day-old glycerin-potato-broth culture (Strain Olsen) which after extraction and concentration had been filtered through paper and then precipitated. It had an antigenic value of 5 e.u. per milligram, and in many other experiments proved to be an excellent preparation. The paratyphoidin-A and -B and the B.-coli extracts were prepared in the same manner. The granular powders were readily dissolved in carbolyzed salt solution, and these made perfectly clear solutions.

The results of the test are summarized in Table 3.

We consider this experiment to be by far the most interesting of our series, in the first place, on account of the low primary toxicity of the preparations, and secondly on account of the marked specificity of the reactions. With the exception of the B.-coli extract, neither preparation gave the slightest reaction in the control rabbits as far as they were not already sensitized by cutaneous tests. Furthermore, the

reactions were well defined in the immunized and infected animals. Some of the reactions were so striking that we could predict the carrier condition, in advance, from the intensity and induration of the areolae. The 24-hour readings (which show, as a rule, greater intensity than the 48-hour readings) were particularly prominent in Rabbits 335, 336, 355, and 361, with 0.02 gm. of typhoidin. In Rabbit 358 and to a certain extent in 361, the response to typhoidin was influenced by the clinical condition of the animal. It is known that during emaciation and anemia, cutaneous tests are frequently diminished in intensity. In most of the clinically healthy rabbits which, as previously stated, later proved to be typhoid carriers, the reaction persisted in a proportionately high degree of intensity for 48 hours. Only Rabbits 355 and 358 were exceptions. In 1 animal (358) this was probably due to an emaciated condition, and, in the others, to some unknown individual factors.

The highly immune rabbits (317, 332, 337) on the other hand, gave smaller, less indurated areolae, which did not persist to the 48th hour. In the quantity of 0.001 gm. the typhoidin P.C.—as was to be expected from its low antigenic value—was not very active. It gave, however, large and indurated areolae in the infected rabbits, and its action as an anaphylactogen was not much impaired apparently, even when the antigenic value promised a different result. The most striking differences between infected and immunized rabbits with a dilution of 1:100 or 0.001 gm. per test were noted at the 24-hour readings.

Rabbit 353, not well immunized, responded poorly to typhoidin. At the 48-hour reading a small red areola with very little induration was present. This negative result was in marked contrast with all the gradations observed in the immune and infected rabbits. From Table 3 it also is apparent how difficult it would be to judge the degree of immunity and the degree of sensitization of a rabbit to *B. typhosus*, from the amount of serum immune bodies. For example: Rabbit 336 showed the same amount of complement-fixing antibodies as 355, yet their skin reactions are different; or, 317 and 335 with the same amount of complement-fixing bodies have an entirely different degree of hypersensitiveness. These observations only confirm our conclusion that no relationship exists between serum immune bodies and the cutaneous response of a rabbit to typhoidin. Some of the carriers and some of the immune rabbits responded to paratyphoid-A and -B and *B.-coli* extracts in quantity of 0.001 gm. The only additional observation with these nonspecific extracts worthy of emphasis is that the

emaciated and sick carriers reacted uniformly to all the extracts. As we know from previous discussions, general hypersensitiveness in some animals is frequently nonspecific as the result of disease or infection.

The results with Group 3 show perfectly that it is possible to detect the carrier state in rabbits by means of the typhoidin test as long as the carrier state is produced by means of intravenous inoculations. Some preliminary experiments on rabbits which had been infected by direct gallbladder-injections showed that the skin reactions are not always as prominent as those noted in Experiment 3 in the rabbits in which the conditions of a human carrier had been imitated as closely as possible. We are at present carefully studying this condition and hope to report some of our observations in the near future.

This experiment also supports our contention that specific cutaneous hypersensitiveness to typhoidin is most marked in an existing typhoid infection of the rabbit. Apparently cutaneous hypersensitiveness in typhoid rabbits varies also directly with the extent and intensity of the disease, as is true of tuberculosis in guinea-pigs. In Rabbit 355 the inflammatory process in the gallbladder was already healing at the time of the test, and therefore the hypersensitiveness was diminished in comparison with that noted in rabbits with flourishing infections. The once-acquired sensitiveness (by infection or artificial means) is probably never entirely lost, tho it may exhibit variations due to factors thus far unknown, which we fear cannot be determined experimentally on rabbits. In our experiments intercurrent infections due to *B. bipolaris-septicus*, for example, were capable of removing the hypersensitiveness entirely. We have thus far been unable to keep infected rabbits for a sufficiently long period (from 1 to 2 years) to test their cutaneous sensitiveness until it entirely disappears.

It would be of the greatest interest to study some human typhoid-carriers and to compare their cutaneous hypersensitiveness to typhoidin with that found in persons infected, reconvalescent, immune, or artificially immunized against typhoid. In using pure typhoprotein and glycerin extracts applied in various dilutions, the problem of cutaneous hypersensitiveness to typhoprotein and its relation to immunity would probably be more satisfactorily solved than has hitherto been the case.

DISCUSSION

The well-known natural immunity of rabbits to an infection with the typhoid bacillus, and the limited possibility of reproducing in this type of animal a disease anatomically and clinically similar to typhoid

fever in man, naturally do not permit of a direct application of our observations to problems of clinical and preventive medicine. And again, the knowledge concerning the mechanism of typhoid immunity in man and animals is so very limited and the present experimental means so crude, that we have to content ourselves with some suggestions which developed from our laboratory observations.

It should be remembered that, aside from morbidity statistics and a few experiments on anthropoid apes, we do not as yet possess means by which we can determine, with certainty, an existing natural or acquired immunity of man or animal against typhoid. Some investigators think that the body fluids, others that the cells of the blood and lymph, or of the tissues most apt to get in contact with the typhoid bacillus, participate in suppressing the fatal multiplication of the invading microorganisms. The conception of a cellular immunity has recently found more and more adherents. The studies of von Wassermann and Sommerfeld¹³ have added considerable support to this conception. These workers found that in mice the well-balanced natural local immunity of the intestinal canal to typhoid bacilli could readily be broken by intercurrent infections or intoxications. These facts lend considerable support to the epidemiologic observations, recently made, that typhoid immunity in man is, in all probability, only relative and that reinfections, with little or no symptoms, are not as rare an occurrence as has been commonly thought to be the case. With typhoid strains highly pathogenic for rabbits, we attempted similar experiments but have thus far only negative results to record.

Our attempts to reproduce in rabbits the mode of typhoid infection in man, by successive passages of the typhoid bacillus through the gallbladders of the animals, are promising in many respects. Until we have succeeded, however, in producing in rabbits a typical intestinal typhoid infection, our methods, which — as already pointed out — are by no means analogous to the conditions in man, have very little value for the determination of the immunizing properties of a vaccine preparation and, in our particular case, for the study of the relation of immunity to the skin test.

The defensive activity of the rabbit following intravenous injection of living typhoid organisms, depends probably to a large extent on the serum immune bodies already present or ready to be mobilized (Bull¹⁴). The absence of a gallbladder carrier state in immunized rabbits subse-

¹³ Med. Klin. 1915, 11, p. 1307.

¹⁴ Jour. Exper. Med., 1916, 23, p. 419; 24, pp. 7, 25.

quent to injection of large amounts of living bacteria of the colon-typhoid-paratyphoid group can not, in our experience, be used as positive evidence of protection. Many factors which we are at present investigating are probably concerned in the pathogenesis of the carrier state, but it is already quite apparent that immunized rabbits develop gallbladder lesions more readily than do normal ones. In having established this fact we were naturally deprived of the most important criterion by which we could judge the immunity of our test animals.

In an endeavor to produce more conclusive results, we employed the fowl-typhoid bacillus as a test organism. The work of Theobald Smith and Ten Broeck,⁶ and our own, has shown that a typhoid-immune rabbit is protected against the toxin of the fowl-typhoid bacillus. Inasmuch as cutaneous hypersensitiveness to fowl typhoidin was demonstrable, we hoped to be able to find some relationship between the degree of the reaction and the resistance of the animals to a subsequent injection of living fowl-typhoid organisms. No parallelisms between skin reaction and resistance to infection could be detected, however. These observations are, in many respects, analogous to an example cited by Nichols.¹⁵ Persons immunized in a prophylactic manner against typhoid will give skin reactions with Paratyphoidin-A and yet possess no immunity to a spontaneous infection.

Whatever method one might choose, therefore, in testing the resistance of a rabbit, one would always produce unnatural conditions which, even if they were conclusive in every respect, would have no bearing on the problem in man. Our findings which show that there does not exist any demonstrable parallelism between cutaneous hypersensitiveness even to typhoidin and the resistance to a subsequent infection, apply therefore only to the rabbit. It is not unlikely that cutaneous hypersensitiveness to typhoidin in man is open to an interpretation different from that which we are able to formulate from our animal experiments.

The fact that infected rabbits react with typhoidin more intensively than immunized rabbits, is quite in accord with the general observations on the tuberculin reaction. Tuberculin hypersensitiveness is a sign of a tubercular infection. For typhoid fever in man, observations are on hand of hypersensitiveness during the acute attack of the disease, but no records could be found of skin tests in carriers. It is to be expected that carriers either give a very marked skin reaction

¹⁵ Jour. Exper. Med., 1915, 22, p. 780.

analogous to our results, or fail to respond on account of absence of antibodies. In case future tests show that the skin reactions in carriers are always positive, we would be justified in concluding that the typhoidin test is a suitable diagnostic agent for the detection of an occult typhoid infection.

This conception is naturally open to criticism, because a gallbladder carrier state develops, as a rule, on the basis of a progressive or already existing immunity and a carrier has a fair degree of resistance to a superinfection even when not always protected against an auto-infection.

Many careful experiments and studies on man must be undertaken before we are prepared to render a final judgment on the merits of the typhoidin test.

Our experiments suggest, however, that careful quantitative tests with pure typhoprotein should be made on carriers of human typhoid. A simple test like the typhoidin test, if it should develop that this test does detect carriers, would be a valuable weapon in the hands of the health officer in the eradication of typhoid fever from communities in which the infection is apt to be perpetuated in this manner.

CONCLUSIONS

A positive typhoidin skin reaction in a rabbit does not indicate that this animal will resist a subsequent intravenous injection of living typhoid bacilli, or that the animal is so protected that it will not become a chronic carrier of bacilli in gallbladder or liver. And again, a marked hypersensitiveness of a typhoid-immune rabbit to fowl typhoidin does not indicate the presence of a resistance to an infection with the same organisms.

No definite relationship exists between agglutinins and complement-fixing antibodies and cutaneous hypersensitiveness to typhoidin and similar extracts. Allergy with bacterial proteins may be demonstrated in rabbits even in the absence of demonstrable immune bodies.

Cutaneous hypersensitiveness to typhoidin is most marked in rabbits infected with typhoid bacilli. Carriers of bacilli in gallbladder and liver develop skin reactions which apparently vary directly with the degree of the inflammatory process in the organs.

THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS
IN GUINEA-PIGS AND RABBITS BY TAURIN,
ALONE AND IN COMBINATION WITH
GOLD CHLORID AND SODIUM
OLEATE *

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Ever since the successful treatment of syphilis by chemotherapy in the hands of Ehrlich and Hata and others, many attempts have been made to affect tuberculosis in a similar manner. Hexheimer, Altmann, and Bernhardt¹ treated lupus with salvarsan with apparently good effect. In 1912 Graf von Linden,² working under the direction of Finckler, tried the efficacy of several copper derivatives in experimental tuberculosis. In her experiments she first employed emulsions of several copper salts, but, finding them strongly irritating, finally utilized an emulsion of copper and lecithin, which not only had no objectionable irritating effects, but seemed to exercise a distinctly curative action on the experimental lesions in guinea-pigs. Similar results were reported by Meissen and Straus,³ who used the same preparation in the treatment of lupus. Bodmer⁴ also reported success in treating a case of human tuberculosis, and Weiss⁵ obtained good results in a single case of tuberculosis of the bladder. Koga⁶ and Otani⁷ have reported favorable results, both in experimental and in clinical tuberculosis, with the use of a preparation of potassium cyanid and copper, to which the name cyanocuprol has been given.

On the other hand, Moewes and Jauer⁸ were unsuccessful in treating human beings with copper preparations, and found them equally ineffective experimentally in guinea-pigs. In previous, unreported experiments I found that copper derivatives had little preventive power

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¹ Jahresb. ü. d. Ergebn. d. Immunitätsf., 1912, 8, p. 13.

² Beit. z. Klin. d. Tuberk., 1912, 23, p. 201.

³ Ibid., p. 223.

⁴ München. med. Wchnschr., 1913, 60,² p. 1758.

⁵ Ibid., 1914, 61,² p. 1558.

⁶ Jour. Exper. Med., 1916, 24, pp. 107, 149.

⁷ Ibid., p. 187.

⁸ München. med. Wchnschr., 1914, 61,¹⁻² p. 1439.

in experimental tuberculosis and no curative value whatsoever. Of 10 guinea-pigs that had been treated with copper emulsion, 7 died within 1 month with characteristic lesions. Corper, DeWitt, and Wells⁹ obtained similar negative results with copper compounds.

Among other metals that have been employed in tuberculosis is gold cyanid, tried by Bruck and Gluck¹⁰ and Hauck¹¹ in lupus with no beneficial effect, but with great danger on account of its toxicity. Mehler and Asher¹² used a substance known as Borcholin or enzytol, which by liberating cholin in the animal body had some apparent effect in surgical tuberculosis. Similar results were obtained with the same preparation by Baisch.¹³

Kahle¹⁴ tried a preparation of silicic acid in guinea-pigs that had been infected with tubercle bacilli, and reports that the lesions became encapsulated and then cicatrized. Noguchi¹⁵ treated tubercle bacilli with sodium oleate and found that such treated microorganisms could be used to produce a certain degree of protection against untreated and virulent cultures of the same strain. Zeuner¹⁶ found the same preparation of value and suggested its use in human cases. Gold and sodium chlorid has been claimed to have some effect on experimental tuberculosis by Gibbs and Shirley.¹⁷

In further, unreported experiments I employed a colloidal mixture of gold chlorid and sodium oleate in experimental tuberculosis in guinea-pigs. When these two substances thus combined were inoculated before the actual infection took place, there seemed to be a distinct inhibition of the disease in the treated animals. Nothing, however, approaching a curative effect after infection had begun could be demonstrated.

Weichardt¹⁸ found that retardin or antikenotoxin extracted from egg albumin had the property of neutralizing toxins of *B. tuberculosis*; and under his guidance Fluhler¹⁹ experimented extensively with this substance in experimental tuberculosis in goats. Control animals succumbed to infection, whereas goats that had been previously treated

⁹ Jour. Am. Med. Assn., 1913, 60, p. 887.

¹⁰ München. med. Wehnschr., 1913, 60,² p. 57.

¹¹ Ibid., p. 1824.

¹² Ibid., p. 748.

¹³ Ibid., 1914, 61,² p. 1613.

¹⁴ Ibid., p. 752.

¹⁵ Centralbl. f. Bakteriöl., I, O., 1909, 52, p. 85.

¹⁶ Ztschr. d. Tuberk., 1913, 20, p. 389.

¹⁷ Quoted in Schumaker's Treatise on Materia Medica and Therapeutics, 1908.

¹⁸ Centralbl. f. Bakteriöl., I, O., 1912, 62, p. 539.

¹⁹ Zentralbl. f. d. ges. Physiol. u. Path. d. Stoffwechs., 1909, 10, p. 564.

with antikenotoxin withstood infection excellently and showed only localized lesions about the point of inoculation. In experiments to which Weichardt has already made reference,²⁰ I found that retardin worked moderately well as a preventive in experimental tuberculosis in guinea-pigs, but had no curative effect.

Another line of possible therapeutic investigation occurred to me, which I believe has not been sufficiently considered. The liver and the bile have, as is known, certain very definite and striking relations to bacterial infections. Posselt²¹ has considered these relations in great detail and drawn the conclusion that the liver in general acts as a filter for bacteria and that the bile has a distinct inhibitive effect on the growth of many microorganisms, tho on bacteria of the colon-typhoid group its action is favorable rather than disadvantageous. Zehden²² some years ago made a very careful study of the literature in reference to liver tuberculosis and was able to come to rather definite and important conclusions. It is evident that tubercles occur with relative infrequency in the liver, and that when they are found there it is probable that they have arisen during the later stages of the disease; in other words, a resistance which previously existed would seem to have been broken down (Dallémagne²³). There is further evidence of a distinct tendency of liver tubercles to heal (Sabourin²⁴). This action of the liver cells on the tubercle bacillus is referable undoubtedly to the bile. Kotlar²⁵ endeavored to find tubercle bacilli in broken-down tubercles which opened into the bile ducts, in vain. Létienne²⁶ was never but once able to find tubercle bacilli microscopically in the bile, and never obtained their growth in cultures. Brissaud and Toupet²⁷ were not able to find tubercle bacilli in the liver of patients dead of the disease. Hanot and Lauth²⁸ found the organisms in experimental tuberculosis in very small numbers in the liver. Maffucci and Sirleo²⁹ found that tubercle bacilli injected through the umbilical cord in embryos or through the portal vein in adults were taken up by the liver cells and destroyed; this action would apparently be due to the presence of the

²⁰ Cited by Schwenk, *Jahresb. ü. d. Ergebn. d. Immunitätsf.*, 1912, 8, p. 44.

²¹ *Ergebn. d. allg. Path.*, 1915, 17, p. 719.

²² *Centralbl. f. allg. Path. u. path. Anat.*, 1897, 8, p. 468.

²³ *Du foie tuberculeux. Thèse d'agrégation, Univ. de Bruxelles.*, Paris, 1891.

²⁴ *Archives de physiol. norm. et path.*, 1883, 15, p. 52.

²⁵ *Ztschr. f. Heilk. Prager Vierteljahrsschrift*, 1894, 15, p. 121.

²⁶ *Arch. de méd. expér. et d'anat. path.*, 1891, 3, p. 761.

²⁷ *Études exp. sous la direct. du prof. Verneuil*, I, Paris, 1887.

²⁸ *Études exp. sous la direct. du prof. Verneuil*, II, Paris, 1888.

²⁹ *Centralbl. f. allg. Path. u. path. Anat.*, 1895, 6, p. 305.

bile within the cell, according to Zehden.²² Kühne suggested the use of taurin in a complicated culture medium for the growth of the tubercle bacillus. Meyer³⁰ found that bovine tubercle bacilli grew well on potato media which contained cattle bile, but not well when mixed with human bile, and that the reverse was also true. Proskauer and Beck,³¹ after careful analysis of these synthetic media for tubercle bacilli suggested by Kühne, found that taurin was not only not necessary, but actually inhibited the growth of tubercle bacilli.

These facts, suggesting some relation between constituents of the bile and a resistance to infection with the tubercle bacillus, led us to incorporate the most characteristic aminoacid of the bile, taurin, $\text{NH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{SO}_2 \cdot \text{OH}$, in a 'combination' treatment, with which the first part of this report deals. In later experiments taurin alone was used, and apparently the encouraging results obtained in our earlier experiments with the combination were due in large part, if not entirely, to the taurin and not to the gold chlorid and sodium oleate combined with it in the earlier experiments.

The materials employed in this investigation were prepared as follows:

1. A colloidal solution of sodium gold chlorid was made by dissolving the pure salt (Kahlbaum or Merck) in distilled water in the proportion of 5 mg. to 100 c.c., and then adding 1 gm. of sodium oleate. The mixture, which forms a purplish colloidal solution, was then sterilized before use.

2. The taurin employed was kindly prepared for us by Dr. K. Miyake in the Rudolph Spreckels physiological laboratory of the University of California. After making vain attempts to synthesize taurin by Strecker's method,³² and having found that the amount recoverable from ox bile was very small, Miyake finally found it could be obtained in quantity from the muscle of shell fish, where it is known to be abundant. For this purpose the common abalone of the Pacific coast, *Haliotis*, proved the best source because of its structure, which is almost entirely muscular. Mendel and Jaffa³³ had already shown that taurin was present in relatively large amounts in the muscle of this animal.

The method finally employed with success and with a relatively large yield of taurin was as follows:

1. Fresh abalone meat is chopped fine in a meat grinder.
2. The ground meat is boiled with a little water in a double boiler for 2 or 3 hours, and the whole boiled contents subjected to pressure to extract the juice.
3. The extracted juice is treated with a saturated aqueous solution of potassium aluminum sulfate, which is added in sufficient quantity to precipitate all the fibrous and albuminous contents. The mixture is allowed to cool and is then filtered through flannel.

³⁰ Diss. Giessen, 1910. Quoted by Cornet and Kossel, *Kolle und Wassermann's Handb. d. pathogen. Mikroorganismen*, 1913, 5, p. 481.

³¹ *Ztschr. f. Hyg. u. Infektionskr.*, 1894, 18, p. 128.

³² *Jour. Am. Chem. Soc.*, 1915, 37, p. 2604.

³³ *Beit. z. chem. Physiol.*, 1903-4, 5, p. 582.

4. To the filtrate is added a saturated solution of barium hydroxid until no further precipitate of sulfates is formed. This precipitate is removed by filtration.

5. The excess of barium in the filtrate is precipitated with CO_2 in the form of barium carbonate. The filtrate is then heated and allowed to stand for from 10 to 12 hours to allow the last trace of barium to be precipitated.

6. The mixture is then filtered, neutralized with acetic acid, and evaporated until taurin begins to crystalize out. Two or three recrystallizations will produce taurin in the form of characteristic crystals, and in relatively pure form.

The first three of the following experiments were carried out in a private laboratory under conditions which precluded more complete study, particularly in reference to the histologic aspects of the problem. In the latter experiments, as will be seen, our observations at autopsy were fully checked by microscopic examination. The method of infection with tubercle bacilli has varied somewhat in the evolution of the work and will therefore be described separately under each experiment.

EXPERIMENT 1

Sixteen guinea-pigs approximately 400 gm. in weight were inoculated subcutaneously over the right pectoral muscle, each with $\frac{1}{16}$ of a 6-months-old culture of bovine tubercle bacillus on glycerin potato (kindly furnished me by Professor Weichardt, of Erlangen, Germany). The whole culture had been suspended in 16 c.c. of sterile physiologic salt solution, rubbed up in a mortar, and shaken in a mechanical apparatus for 2 hours until homogeneous. Each dose comprised a volume of 1 c.c. The inoculated animals were then divided into 3 series.

Series 1. On Oct. 19, 1913, 24 days after inoculation, the treatment of 6 animals (3, 5, 16, 18, 19, and 25) was begun and carried out as follows. The treatment lasted for 48 days and on every 2nd or 3rd day a subcutaneous injection either of taurin (dose 0.05 or 0.1 gm.) alone or of a combination of taurin, 50 mg., and of gold chlorid, 0.5 mg., plus sodium oleate, 10 mg., injected successively, was given. In the period of treatment the total amount of taurin alone given in 14 doses was 1.225 gm., and the total amounts of the substances given in combination were—taurin, 250 mg.; sodium oleate, 50 mg., and gold chlorid, 2.5 mg.

Series 2. In 2 animals (11 and 31) treatment was begun 40 days after inoculation. Nine injections only were given.

Series 3. This series, comprising 8 animals (1, 2, 6, 7, 28, 35, 37, and 39), was left untreated as controls.

The results are summarized in Table 1.

TAURIN IN TREATMENT OF EXPERIMENTAL TUBERCULOSIS 447

TABLE 1
RESULTS OF EXPERIMENT 1

Guinea-Pig	Day of Death	Weight (gm.) — = Loss. + = Gain		Postmortem Examination
SERIES 3. CONTROLS				
2 37 28	2nd 2nd 31st	— 20 — 40	Av. loss 21 gm.	No distinctive lesions No distinctive lesions Lung showed diffuse tuberculous areas. Liver and spleen were enlarged, contained many tubercles. Axillary lymph nodes enlarged and caseated
6	43rd	— 55		Nodules of lung less diffuse than in Guinea-pig 28. Liver, spleen, and lymph nodes similar to those organs in 28
1 7 39	45th 45th 64th	— 30 — 40 + 20		Similar to Guinea-pig 28 Similar to Guinea-pig 28 Lung full of diffuse tuberculous areas. Liver, spleen, and lymph nodes contained many large tuberculous nodules, which were caseated
35	Killed on 85th	+ 15		Similar to Guinea-pig 39
SERIES 2 (TREATED OVER A SHORTER PERIOD)				
31	63rd	+ 35	Av. gain 80 gm.	Lung contained good-sized but well-localized tubercles. Liver enlarged; nodules whitish and well localized. Spleen full of many small-sized nodules. Lymph nodes enlarged and caseated
11	Killed on 85th	+125		Few well-localized nodules in lung only
SERIES 1 (TREATED OVER A LONGER PERIOD)				
3	Killed on 38th	— 25	Av. gain 37 gm.	Small distinct nodules in lung. Nodules in liver and spleen small but definite. Axillary lymph nodes enlarged and caseated
5 18	45th 52nd	+ 60 + 15		Similar to Guinea-pig 3 Lung contained good-sized but localized nodules. Liver enlarged; nodules whitish and well localized. Spleen full of many small-sized nodules. Lymph nodes enlarged and caseated.
25	Killed on 61st	+ 15		Lung contained white well-localized nodules in small numbers. Liver and spleen enlarged, containing a few tubercles. Lymph nodes enlarged, with a small area of caseation
16	Killed on 85th	+ 90		Surface of the lung for the most part smooth. A few discrete nodules visible on cut surface. Surface of liver cirrhotic. Definite tubercles on surface were rare, and well localized
19	Killed on 85th	+ 70		Lung contained many small nodules on the surface, which were well localized. Liver surface for the most part smooth. Spleen showed puckered discrete nodules of varying size; not caseated. On section, well localized

Among the controls all the animals save one died within 64 days, and 5 of these 7 died of tuberculosis. The average loss of weight in these animals and the one that was killed was over 23 gm. In the treated series No. 1, only 2 animals died in the first 60 days; one was killed during this period; and the other three were killed subsequently to observe any change in the lesions. At the time of death or killing the average gain in weight for this series was over 37 gm., as contrasted with the average loss in the other series. In the treated series No. 3, there were only 2 animals, one of which died and one of which was killed after 85 days; the average gain in these two animals was 80 gm. The postmortem findings in a general way would seem to indicate an arresting of the process, and in some instances an attempt at repair; but microscopic examination would be necessary in detail to determine this point.

EXPERIMENT 2

This experiment was carried out in a manner similar to that of Experiment 1, with the exception that a human strain of tubercle bacillus (obtained from the Cutter laboratory, Berkeley) was employed. Twenty guinea-pigs, weighing on the average 350 gm., were inoculated subcutaneously with $\frac{1}{38}$ of an old culture of *B. tuberculosis* (humanis) suspended in 1 c.c. of salt solution. The animals were then divided into 4 groups of 5 each, and 1 group was reserved as control without treatment. The outline of treatment for the other three groups follows.

Series 3. Treatment was begun 7 days after inoculation and lasted 3 months; during this period 11 injections of taurin alone in a dose of from 0.1 to 0.15 gm., and 7 injections of taurin plus the combination of gold chlorid, 0.4 mg., and sodium oleate, 10 mg., were given.

Series 2. Treatment was begun 14 days after inoculation and lasted 3 months less a few days; 15 injections of taurin alone were given and 4 injections of a combination of taurin and gold chlorid and sodium oleate in amounts similar to those of Series 3.

Series 1. Treatment was begun 21 days after inoculation, and lasted 10 weeks, during which 14 injections of taurin were given (0.1 to 0.15 gm.), and 4 injections of taurin plus the combination of gold chlorid and sodium oleate, as in the other groups. The results of this experiment are expressed in Table 2.

It would seem evident from this experiment that not only was the process arrested, but, judged from the weights and gross postmortem findings, all the animals in Series 3 and 2 were nearly or entirely cured

TABLE 2
RESULTS OF EXPERIMENT 2

Guinea-Pig	Day of Death	Weight (gm.) — = Loss. + = Gain		Postmortem Examination
CONTROLS				
150	28th	— 85	Av. loss 17 gm.	Lung, liver, and spleen contained many small tubercles. Lymphatic glands enlarged and caseated Lung full of large diffuse tuberculous masses. Liver and spleen enlarged, containing many tubercles. Lymphatic glands enlarged and caseated
142	61st	+ 45		
141	66th	— 10		
144	70th	— 45		
148	79th	+ 10		
SERIES 3 (TREATED EARLIEST AND LONGEST)				
41	Killed on 82nd	+105	Av. gain 137 gm.	Lung showed small whitish well-localized tubercles. Liver and spleen were enlarged, and contained many small but localized nodules. Lymphatic glands enlarged and caseated Lung, liver, and spleen similar to Guinea-pig 41's. Lymphatic glands enlarged but very slightly caseated Lymph nodes enlarged and hardened, but not caseated. Lung and liver showed no definite tubercles. Spleen still contained definite tubercles
57	Accidentally died on 106th	+ 70		
62	Killed on 108th	+185		
75		+205		
64		+120		
SERIES 2 (TREATED LATER THAN SERIES 1 BUT FOR SAME PERIOD)				
53	67th	+ 10	Av. gain 169 gm.	Lung showed good-sized whitish nodes. Liver and spleen enlarged, but no definite nodules visible. Lymphatic glands enlarged and hardened, but not caseated Lung had a few well-localized nodes. Liver enlarged; no definite nodes. Lymphatic glands enlarged Lung and liver contained a few well-localized whitish tubercles. Spleen—no definite tubercles visible. Lymphatic glands enlarged No definite nodes in lung, liver, or spleen Similar to Guinea-pig 52
42	Killed on 108th	+310		
49	Killed on 108th	+ 65		
52	Killed on 108th	+200		
63	Killed on 108th	+266		
SERIES 1 (TREATED LAST AND SHORTEST PERIOD)				
143	76th	+ 60	Av. gain 94 gm.	Lung contained whitish well-localized tubercles. Liver was cirrhotic and contained a few tubercles. Lymphatic glands enlarged Lung contained cavity with indurated wall. Liver was enlarged and contained a few tubercles. Spleen enlarged, no tubercles. Lymph nodes enlarged Lung—a few well-circumscribed nodules with small cavity with indurated walls. Liver and spleen were enlarged and contained numerous tubercles. Lymphatic glands enlarged and caseated Lung—well-circumscribed tubercles and a cavity. Liver and spleen were enlarged and contained a few tubercles. Lymphatic glands enlarged and indurated No definite nodules found in lung, liver, or spleen. Lymph nodes enlarged and hardened
135	106th	+ 55		
133	Killed on 108th	+ 95		
140	Killed on 108th	+ 95		
145	Killed on 108th	+165		

of active tuberculosis. Whether or not living tubercle bacilli still remained in the lesions was not determined. The animals in Series 1 apparently were almost as favorably affected as those in the earlier series, tho the lesions, as indicated by cavity-formation, had proceeded further before the treatment was begun.

It would appear, then, that under the conditions mentioned, which were not so exactly determined as would be desirable in point of view of dosage, guinea-pigs were cured by the combined treatment after a subcutaneous inoculation of either bovine or human tubercle bacilli.

EXPERIMENT 3

In this experiment a modification was made of the experiments already described in that the guinea-pigs were inoculated intraperitoneally with a human culture similar to the one employed in Experiment 2 (obtained from the Cutter laboratory). Seventeen guinea-pigs

TABLE 3
RESULTS OF EXPERIMENT 3

Guinea-Pig	Day of Death	Weight (gm.) — = Loss. + = Gain		Postmortem Examination
CONTROLS				
32	63rd	— 80	Av. loss 142 gm.	Lung, liver, spleen, peritoneum, and omentum, all show many diffuse tubercles. Lymph nodes enlarged and caseated
17	67th	—350		
21	72nd	—145		Same as Guinea-pig 33
18	78th	— 65		
20	78th	—165		
30	78th	— 10		
19	90th	—180		
TREATED ANIMALS				
24	75th	—180	Av. loss 49.5 gm.	Lung—many discrete nodules. Liver and spleen enlarged; no definite nodules visible. Peritoneum—no nodules visible. Lymph nodes enlarged, not caseated
34	81st	—125		Similar to Guinea-pig 24, except that inguinal and mesenteric lymph nodes were partly caseated
23		—195		Lung—very few tubercles remain. Liver—a few discrete tubercles. No tubercles found in spleen or peritoneum. Lymph nodes enlarged, not caseated
22	Killed on 90th	+ 20		Similar to Guinea-pig 23
27		—110		Guinea-pigs 27, 29, and 25 similar to Guinea-pig 24, but lymphatic glands showed a few central caseated areas
29		—125		
25		+ 35		
26		+ 35		Guinea-pigs 26, 31, and 32 had lesions even less marked than those in the preceding
31		+ 50		
32		+100		

were given intraperitoneal injections of 1 c.c. of a suspension of tubercle bacilli containing from 5 to 17 c.c. Seven of the animals were left as controls without treatment. Fourteen days later injections of taurin were begun subcutaneously in the remaining 10 animals. Sixteen of these injections were given in a dosage of from 0.1 to 0.2 gm. every 3 days and were followed by 8 injections of taurin plus gold chlorid (0.2 mg.) and sodium oleate (10 mg.).

The results of the experiment are summarized in Table 3.

It will be observed in this experiment that with one exception the controls all died before the 90th day, whereas only 2 of 10 treated animals died during this period. Among the remaining 8 animals, 5 showed an increase in weight in contrast with the regular decrease in controls. The lesions in the treated animals contrast markedly with those in the untreated animals.

It occurred to us at this point in our work that the results we had produced might be due to taurin alone, instead of taurin in combination with the other substances, and the next experiment (Experiment 4) was designed to test what result in treatment could be produced with taurin alone. Unfortunately, the dose employed in this experiment was the same as had been employed in Experiment 2, and when administered intraperitoneally it led to death so rapidly in the controls that the curative effect of the taurin alone or in combination was not as evident as in the last experiment.

EXPERIMENT 4

The same emulsion of tubercle bacilli suspended in sterile salt solution which had been employed in the second experiment and which had been kept in the icebox about 2 weeks, on April 2, 1914, was inoculated into 16 guinea-pigs and in the same dose, that is to say, approximately $\frac{1}{3}$ s of a culture, but instead of subcutaneously as in the earlier experiment, they were inoculated directly in the peritoneal cavity. They were then subdivided into 2 groups of 8 each, 1 of which was kept untreated as a control. The 2nd group were treated for the most part with taurin alone, but a few of the animals toward the end were given from 2 to 4 doses of the gold-chlorid sodium-oleate combination in addition. The average weight of these animals was 450 gm. As the treatment differed somewhat, they had best be considered separately. The results of the experiment are expressed in Table 4.

In this experiment the control animals died more rapidly than in previous experiments, owing to the large intraperitoneal injections,

TABLE 4
 RESULTS OF EXPERIMENT 4

Guinea-Pig	No. of Injections	Time of Treatment	Day of Death	Weight (gm.) — = Loss + = Gain	Postmortem Examination
7)			41st	—120	Guinea-pigs 7, 3, 1, and 2 all showed diffuse tuberculous peritonitis with tubercles covering peritoneum and omentum, diffuse nodes of the lung, liver and spleen enlarged, with small tubercles
3			45th	— 95	
1			47th	—130 Av.	
2 Controls			47th	—105 loss	
11			48th	— 55/106	
5			50th	—145 gm.	
4			56th	— 80	Guinea-pigs 11, 5, 4, and 6 showed conditions in the organs essentially similar to those described in the first group, except that in 5 and 11 peritonitis was not marked. Lymph nodes enlarged and caseated in all 5 animals
6)			56th	—115	
10	11 (.12—.15 gm. taurin)	1 mo.	52nd	—120	Guinea-pigs 10, 16, 9, 8, 15, and 13 all showed a moderate number of tubercles in lung, liver, and spleen, but few on peritoneum and omentum. Lymphatic glands caseated only in Nos. 10 and 15, in the glands of which small central areas were softened
16	14 (taurin).....		57th	+ 30	
9	11 (.12—.15 gm. taurin)	1 mo.	61st	— 40	
8	15 (taurin).....	7 wk.	61st	—130	
15	16 (taurin).....	7 wk.	62nd	—110	
13	16 (taurin and 2 combination treatments)		64th	—135 Av. loss 19 gm.	
14	16 (taurin and 4 combination treatments)		Killed on 88th	+210	Guinea-pigs 12 and 14 showed no tubercles in lung, liver, spleen, or peritoneum. Lymph glands enlarged and hardened
12	16 (taurin and 4 combination treatments)		Killed on 88th	+140	

and all showed diffuse lesions, including tuberculous peritonitis. The controls had all died before the treated animals, with one exception (treated animal No. 10). All the controls showed a marked loss of weight, whereas 3 of the treated animals actually gained in weight, in spite of the death of one of them (16). The appearance of the treated animals post mortem also differed distinctly from that of the untreated animals. Two animals (12 and 14) appeared to have been cured of the more rapid infection.

In the subsequent experiments to be reported at this time rabbits instead of guinea-pigs were used, and they were infected with bovine tubercle bacilli.

EXPERIMENT 5

Six rabbits, averaging in weight about 2000 gm., were given intravenous injections of 10 mg. each of a culture of *Bacillus tuberculosis bovinus*, designated G, grown 6 months on glycerin broth (isolated

TABLE 5
RESULTS OF EXPERIMENT 5

Rab- bit	No. of Injections	Day of Death	Weight (gm.) — = Loss + = Gain	Postmortem Examination
CONTROLS				
684		44th	-450	Lungs filled with diffuse masses of tubercles which microscopically showed necrotic areas and lymphoid, epithelioid, and giant cells in considerable numbers. Liver appeared normal in gross, but in microscopic section showed small well-localized tubercles containing epithelioid and lymphoid cells with some central necrosis in places. Spleen appeared normal both in gross and on microscopic examination. Lymph nodes enlarged and caseated.
659	Controls	63rd	-450	Lung full of diffuse tubercles. Practically no crepitation. Microscopically, it showed many diffuse necrotic areas with lymphoid, epithelioid, and giant cells. Spleen was enlarged and congested, and showed a few areas of epithelioid and lymphoid cells, without distinct caseation. Liver apparently normal. Microscopically, it was congested, but without tubercles. Kidney showed a few discrete tubercles near cortex, containing epithelioid and lymphoid cells. Lymph glands enlarged with areas of caseation.
633		71st	-250	Liver apparently normal in the gross, but on section it contained small areas of lymphoid and epithelioid cells. Kidney contained small tubercles with many epithelioid and a few lymphoid cells. Peritoneal cavity contained 50 c.c. of exudate and many tubercles. Lung filled with large masses of caseated diffuse tubercles with large necrotic areas and lymphoid, epithelioid, and many giant cells. Tubercle bacilli could be stained in the necrotic areas. Spleen enlarged; no tuberculosis.
645	31 (taurin).	Killed on 44th	+400	Liver normal. Lung showed a few very discrete areas containing large epithelioid and plasma cells surrounded by lymphoid cells. No tubercle bacilli found in these areas. Spleen and mesenteric lymph nodes apparently normal.
601	40 (taurin).	78th	+100	Lung revealed a very few well-localized nodes similar to those of the last animal. Lower left lobe showed gangrene. Liver apparently normal in the gross. On section numerous tubercles found, with areas of necrosis containing epithelioid, lymphoid, and giant cells. Coccidiosis. Some of the areas of necrosis surrounded by young connective-tissue cells. Kidney normal. Spleen and lymph nodes apparently normal.
672	43 (taurin).	Killed on 78th	+700	Lung contained numerous well-circumscribed nodules with slight areas of caseation. No tubercle bacilli found. Some of these areas invaded by fairly dense and recent connective tissue. Kidney normal. Liver congested, but normal.

from tuberculous glands in 1911 by Dr. Paul Lewis, of Philadelphia). Three days after inoculation the treatment of 3 animals (601, 645, and 672) was begun; they were given from 31 to 43 doses of 0.5 gm. each of taurin dissolved in 7.5 c.c. of distilled water, every day, over a period of about 7 weeks. The result of this experiment is briefly outlined in Table 5.

In this experiment only one of the treated animals died, whereas all the controls died. The one treated animal which died (601) contained coccidiosis, and the evidence of tuberculosis was not marked. The lesions in the lungs showed very striking contrasts in the control animal that died on the 44th day, as compared with the one killed at that period (684 and 645). It was impossible, however, to demonstrate tubercle bacilli in the lesions of the two treated animals (601 and 645), and only a very few were seen in the third treated animal (672), whereas they were evident in numbers in the untreated controls. Altho the experiment contains few animals, it gives distinct evidence of the arrest and beginning of repair of the tubercles.

EXPERIMENT 6

In the final experiment, 20 rabbits, averaging about 1600 gm. in weight, were given intraperitoneal injections of 1 mg. of the same culture used in the last experiment, bovine bacillus G, from the surface of a glycerin broth culture aged 11 weeks, and were divided into 4 series, one of which was left as a control.

In the 2nd series treatment was begun 3 days after inoculation, in the 3rd series 7 days after, and in the 4th series 14 days after. The 2nd series was given 28 injections of 0.5 gm. of taurin intravenously on alternate days, and the 3rd and 4th series 28 injections of the same dosage and frequency. The number of doses was somewhat less in the few animals that died during the course of the experiment. The results of this experiment are tabulated in Table 6.

In this experiment, then, it is evident that the rabbits were in nearly every instance completely cured of an infection which was fatal to the controls in all but a single instance (786). This last animal showed advanced lesions of the lungs, tho it had survived to 143 days and had actually gained in weight.

SUMMARY AND CONCLUSIONS

This report deals with the curative effect of taurin alone or in combination with a colloidal mixture of sodium gold chlorid and

TAURIN IN TREATMENT OF EXPERIMENTAL TUBERCULOSIS 455

TABLE 6
RESULTS OF EXPERIMENT 6

Rabbit	Day of Death	Weight (gm.) — = Loss. + = Gain	Postmortem Examination
SERIES 1. CONTROLS			
773	19th	—750	<p>Small tubercles of the lung and peritoneum. Retroperitoneal lymph glands enlarged. Spleen and liver normal. Rabbits 782 and 787—similar lesions. Lung contained many diffuse tubercles, and necrotic areas with giant and lymphoid cells and numerous tubercle bacilli. Liver—no marked change. Spleen enlarged without visible tubercles. Peritoneum rough with small tubercles. Lymph nodes enlarged and caseated. Lung contained many diffuse tubercles with caseation. Lymph nodes enlarged and caseated. Peritoneum roughened. Spleen and liver showed no tubercles. Similar to Rabbit 789</p>
782	50th	—100	
787	50th	—400	
		Av. loss 200 gm.	
780	141st	— 50	
786	Killed on 143rd	+300	
SERIES 2. TREATED ANIMALS			
772	50th	—800	<p>Peritoneum was rough and contained 30 c.c. clear exudate. Retroperitoneal lymph glands somewhat enlarged. All other organs apparently normal. Rabbits 769, 815, and 816 showed an absolutely normal appearance, with the exception of slightly enlarged lymph nodes. No foci of tuberculosis found microscopically. Lung contained a few well-localized tubercles, which showed thickened alveolus septa, and a small focus infiltrated with lymphoid cells. No tubercle bacilli found in lesion</p>
769	Killed on 156th	+500	
815		+500	
816		+450	
790		+450	
		Av. gain 220 gm.	
SERIES 3			
783	99th	—800	<p>Lung showed a small focus of pneumonia, but no definite nodules. Liver showed two areas of coccidiosis. Kidney and spleen congested; no tubercles. No lesions visible in these animals</p>
771	Killed on 156th	+200	
777		+200	
773		+150	
774		+450	
		Av. gain 40 gm.	
SERIES 4			
776	99th	—250	<p>Showed a pneumonic area in one lung. Organs otherwise normal. These animals showed no lesions. Retroperitoneal lymph glands somewhat enlarged, but not caseated</p>
784	Killed on 156th	+150	
759		+200	
781		+100	
W		+100	
		Av. gain 60 gm.	

sodium oleate in experimental tuberculosis in guinea-pigs and rabbits. Apparently as favorable results were obtained with taurin alone as with taurin combined with sodium gold chlorid and sodium oleate. From preliminary tests taurin may be injected in as large and probably in much larger doses than were employed, either subcutaneously or intravenously, without any symptomatic disturbance.

Guinea-pigs were infected with either bovine or human strains of tubercle bacilli. The results were similar with both. In nearly every instance the controls all died before any of the treated animals, and whereas the controls lost weight almost uniformly, the treated animals gained. The majority of the treated animals were killed for observation many days after the controls had all died. The contrast in the extent of visible tuberculosis between controls and treated animals was marked. Whereas the process was advanced in the controls, it was arrested and in some instances apparently cured in the treated animals. The success of the treatment varied naturally in accordance with the variations in infecting dose, the route of inoculation, and the time of beginning treatment, as specified in the protocols. In all experiments and in nearly every instance the results were distinct, and in many instances startling. The process of the disease was arrested and apparently cured when treatment was begun as late as 3 weeks after infection.

Rabbits were infected with intraperitoneal or intravenous injections of bovine tubercle bacilli and treatment was carried out by intravenous injections of taurin alone. The results were similar, but even more marked than in guinea-pigs. Otherwise fatal infections, which in evolution were slower than in guinea-pigs, were not only arrested, but evidence of them often definitely disappeared. Histologic examinations of organs in these experiments showed in treated animals arrested tubercles, absence of caseation, disappearance of tubercle bacilli in the lesions, and evidence of repair by connective-tissue ingrowth, as controlled by the advancing lesions, with caseation and numerous bacilli in the controls. A number of treated animals showed minimal evidences of tuberculosis, and in no inconsiderable number the tissues were essentially normal microscopically. In such successful cases treatment was begun as late as 2 weeks after inoculation.

THE DIFFERENTIATION OF THE PARATYPHOID-ENTERITIDIS GROUP. I*

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Study of the large group of bacteria represented by *B. paratyphosus*, *B. enteritidis* (Gärtner's bacillus), and *B. suipestifer* has not yet resulted in a unified opinion. The relative importance of diagnostic characters, the cultural and pathogenic interrelationships between the different members of the group, the extent to which variation occurs, and the distribution of the several types in nature are questions on which there is no general agreement. Some observers hold that the bacillus associated with many cases of human paratyphoid fever (*B. paratyphosus* B) cannot be distinguished from a similar bacillus found in certain epidemics of meat poisoning or from the bacillus commonly present in the bodies of swine dying from hog cholera (*B. suipestifer*) (Sobernheim;¹ Weber and Haendel²). Others maintain that a clear differentiation into two types is possible on the basis of certain fermentative or serologic tests and that such a distinction corresponds with the known facts of epidemiology and distribution (Bainbridge,³ Savage⁴). On the practical side it is important to know whether human paratyphoid fever is due to infection from human sources (like typhoid fever) or from the lower animals (as is apparently true of certain 'food-poisoning' outbreaks). These considerations emphasize the necessity for better methods of differential diagnosis or failing that, a more complete knowledge of the extent and frequency of significant variation.

With this end in view, I have made a comparative study of a considerable number of microorganisms belonging to this group, and have attempted especially to determine possible correlations between agglutination reactions and differences in fermentative power and other biologic qualities. The cultures have been kept under observation for some time and tested at frequent intervals. All those here considered are of human or porcine origin. A number of them have been in my possession several years, while others are recently isolated.

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¹ Hyg. Rundschau, 1912, 22, pp. 953, 1019.

² Berl. klin. Wchnschr., 1912, 49, p. 2205.

³ Lancet, 1912, 1, p. 705.

⁴ Jour. Hyg., 1912, 12, p. 1.

The cultures have been obtained from various sources and I am indebted to many friends and correspondents for their courtesy. In such studies I believe that especial importance attaches to those cultures whose source and date of isolation can be exactly ascertained.

The methods used, where not otherwise stated, have conformed to the 'Standard Methods' of the American Public Health Association. The cultures received have been subjected to the following routine procedure: (a) Plating on Endo medium. (b) If colonies are all alike and conform to the paratyphoid type, 2 are transferred to agar. (c) The three strains—the original culture, and the two from single colonies—are inoculated into dextrose and lactose broth, litmus milk, and gelatin and tested for indol-production. (d) Only those strains are included in this study which, in the media mentioned, yield reactions that are typical and uniform.

The following table summarizes the list of cultures studied. Complete details as to source of isolation are given in the lists at the end of this paper.

Cultures from human sources:

- (A1) Stock cultures from laboratory collections: 4, 11, 48, 229.
- (A2) Isolated from blood in human paratyphoid fever: 3, 9, 131, 158, 188, 191, 198.
- (A3) Isolated from feces in human paratyphoid fever: 212, 213, 214, 215, 216, 217, 218, 219, 230.
- (B1) Stock cultures from laboratory collections: 2, 5, 223.
- (B2) Isolated from blood in human paratyphoid fever: 12, 130, 203, 209, 210.
- (B3) Isolated from feces in human paratyphoid fever: 47, 149, 150, 179, 211, 221,* 225.
- (B4) Isolated from human gallbladder: 8, 202.
- (B5) Isolated from organs in fatal cases of food poisoning: 151, 152, 222, 224.
- (B6) Isolated from lymph-gland infection in man: 185.
- (E1) Stock cultures from laboratory collections: 50, 51, 52.
- (E2) Isolated from human feces: 53, 206.
- (E3) Isolated from organs in fatal case of food poisoning: 228.

Culture from food materials implicated in food-poisoning outbreak: B 180.

* From a 'carrier.'

Cultures from diseased swine:

- (S1) Stock cultures of 'hog-cholera bacilli' from various laboratory collections, exact sources and date of isolation unknown: 62, 114, 115, 160, 161.
- (S2) From organs of diseased swine; full information regarding isolation: 63, 118, 132, 133, 134, 162, 163, 165, 167, 168, 169, 170, 171, 172, 173, 174, 175, 177, 178, 234.

In addition to the strains here enumerated, many stock cultures have been sent me which from the histories given seem to be from the same original source. If the reactions were identical, the duplicate cultures were discarded, and the results do not appear in these tabulations. Thus several cultures, apparently subcultures of No. 5 and identical with it, were sent me from different laboratories; only No. 5 is referred to in this paper. Fourteen strains from the same case as No. 12, isolated from blood, bile, and feces at different times gave identical reactions with it, and are not included in the final tabulation here presented. On the other hand, Nos. 212-219, tho isolated from different cases, are all from a single paratyphoid-A epidemic and are very likely all offshoots of the strain causing the outbreak. In all, about 200 cultures have been studied.

Three groups of the cultures are from human sources. These are *B. paratyphosus* A (20 strains), *B. enteritidis*, Gärtner (6 strains), and *B. paratyphosus* B (22 strains). In addition I have had under observation 1 culture (para-B type) from food material implicated in food poisoning and 25 from diseased swine, mostly from fatal 'hog-cholera' cases. These 74 cultures are alike in some of their cultural characters, but divergent in others. All are motile, gram-negative bacilli. Their growth on agar is more luxuriant as a rule than that of the typhoid bacilli, but less luxuriant than that of *B. coli*. The colonies on Endo plates vary considerably in size, and some strains show much more pink color than others. Gelatin is not liquefied by any of the strains in a 6-weeks' test.

LITMUS MILK

Milk or whey tinged with litmus has long been recognized as a useful medium in the differentiation of this group. Inoculation with strains of *B. paratyphosus* B, *B. enteritidis*, and *B. suipestifer*, at 37 C., is followed by an initial acidity which may last 2 or 3 days or longer, but which then gradually changes to an alkaline reaction. Strains of

B. paratyphosus A, on the other hand, give rise to an acid reaction which as a rule is much more persistent. Continued acid reaction has been commonly regarded as the chief cultural character separating *B. paratyphosus* A from the other groups.

Some European workers have preferred to use litmus whey rather than litmus milk for this test, but the latter is more easily prepared, and in comparative tests I have never been able to observe that whey presents any advantages over the whole milk. This is also the experience of other observers.⁵ The medium I have employed is certified milk allowed to stand and separated from the cream by siphon; to this, 7% of a 1% solution of Merck's litmus is added. The tubes so prepared are heated in the Arnold sterilizer for 20 minutes on 3 consecutive days.

The results are as follows: The freshly isolated cultures of *B. paratyphosus* B, *B. supestifer*, and *B. enteritidis* produce at first a slight acidity, but this soon gives way — usually within 4 days — to an alkaline reaction, which increases in intensity for a week or more. Strains that have been long under cultivation often show a slower rate of increase in alkalinity. Considerable 'irregularity' is observed in certain strains.

The *paratyphosus*-A cultures remain acid as a rule much longer than those of the other group. Some writers⁶ have referred to the acidity as 'permanent,' but fail to state the exact period of observation. A number of investigators,⁷ on the other hand, have recognized that alkali-production takes place in some cultures of the A type, albeit much later than in the other group. Bainbridge says: "*B. paratyphoid* A as a rule produces permanent acidity in litmus milk, though occasionally milk may become alkaline after the lapse of several weeks." Bradley especially emphasizes the fact that "the subdivision into A and B types [on the basis of milk reaction] is one of degree, and cannot be maintained biochemically." Krumwiede and his coadjutors also record observations showing that *paratyphoid*-A strains sooner or later produce alkali in milk, and express the opinion that behavior in this medium cannot be strictly used as a qualitative method of differentiation.

⁵ Buxton, *Jour. Med. Research*, 1902, 3, p. 201. Krumwiede, Pratt, and Kohn, *ibid.*, 1916, 30, p. 55.

⁶ See, for example, J. Henderson Smith, *Brit. Med. Jour.*, 1915, 2, p. 1.

⁷ Libman, *Jour. Med. Research*, 1902, 3, p. 168. Buxton, *ibid.*, p. 201. Boycott, *Jour. Hyg.*, 1906, 6, p. 33. Bainbridge, *Jour. Path. and Bacteriol.*, 1909, 13, p. 443. Bradley, *Jour. and Proc. Roy. Soc. N. S. Wales*, 1912, 46, p. 74. Krumwiede, Pratt, and Kohn, *Jour. Med. Research*, 1916, 30, p. 55.

My own observations show that there are considerable differences in the speed and degree of alkali-formation among the members of the paratyphoid-B-enteritidis-suipestifer group, so that a series of cultures may be arranged in gradation. All the cultures of *B. paratyphosus* A in my possession have produced alkali in litmus milk after varying periods of time. Every strain has been tested in this medium, but only one series need be reported in detail here, as it is entirely typical of the whole collection.

TABLE 1
REACTION OF STRAINS OF THE PARATYPHOID-B-ENTERITIDIS-SUIPESTIFER GROUP IN
LITMUS MILK

Days	More Acid Than Control		Like Control		More Alkaline Than Control	
	Type	No. of Strains	Type	No. of Strains	Type	No. of Strains
2	Para A Para B B. enteritidis	8 16 5	Para B B. enteritidis	1 3		0
4	Para A	8	Para B B. enteritidis	1 1	Para B B. enteritidis	16 7
7	Para A	8		0	Para B B. enteritidis	17 8
14	Para A	2	Para A	6	Para B B. enteritidis	17 8
30	Para A	1			All others more alkaline than control	

Arranged in order of increasing alkalinity, changes are observed in the relative position of cultures (all kept in the same rack at 37 C. under identical conditions). If the strains are recorded in gradation — No. 1 representing the most acid, and No. 33 the most alkaline, after 4 days' growth — the order after 7 days is as follows: A3, A7, A1, A5, A2, A8, A6, A4, B11, B13, B10, B12, E9, B20, B16, E23, E15, B14, B22, E25, B19, B27, B21, B24, E30, E17, E28, E32, B18, B26, B31, E33, B29; in 14 days: A7, A1, A2, A3, A8, A4, A6, A5, B11, B10, E9, B12, B13, B14, E23, E15, B21, B27, E25, B24, E30, B22, E17, E28, B16, B20, B19, E32, B29, E33, B26, B18, B31; and in 30 days: A1, A8, A6, A7, A5, A2, A4, A3, B11, E15, B10, E32, B21, B12, B27, B14, E28, E30, E23, B13, E9, E25, B24, E17, E33, B18, B31, B16, B22, B29, B20, B19, B26.

The sort of deviation observed in a series of paratyphoid-A strains is shown in Table 2. Alkali-formation is first observable after about

2 weeks and then proceeds much more rapidly in some strains than in others. There is often a difference in the amount and rapidity of alkali-formation in strains obtained from single colonies after plating an apparently pure culture on Endo medium (see Nos. 215 and 219, Table 2).

TABLE 2

Strain	Reaction in Litmus Milk after					
	24 Hours	48 Hours	5 Days	11 Days	15 Days	30 Days
212	A	A	A	A	SA	Alk
212 C1	A	A	A	A	SA	Alk
212 C2	A	A	A	A	SA	Alk
213	A	A	A	A	SA	Alk
213 C1	A	A	A	A	SA	Alk
213 C2	A	A	A	A	SA	Alk
214	A	A	A	A	SA	Alk
214 C1	A	A	A	A	SA	Alk
214 C2	A	A	A	A	SA	Alk†
215	A	A	A	A	SA	Alk
215 C1	A	A	A	A	SA	Alk
215 C2	A	A	A	A	C	Alk
216	A	A	A	A	SA	Alk†
216 C1	A	A	A	A	SA	Alk†
216 C2	A	A	A	A	SA	Alk†
217	A	A	A	A	SA	Alk
217 C1	A	A	A	A	SA	Alk
217 C2	A	A	A	A	SA	Alk
218	A	A	A	A	SA	Alk
218 C1	A	A	A	A	SA	Alk
218 C2	A	A	A	A	SA	Alk
219	A	A	A	A	SA	Alk
219 C1*	A	A	A	A	SA	Alk†
219 C2	A	A	A	A	SA	Alk

A = more acid than control.

SA = slightly acid.

C = like control.

Alk = more alkaline than control.

* 219 C1 decidedly more alkaline in 20 days than any other.

† Decidedly more alkaline than others, which are all approximately the same.

A comparison has been made between the rate of alkali-formation in litmus milk in a shallow layer in Erlenmeyer flasks (0.5 cm.) and that in test tubes one-half to two-thirds filled (about 8 cm.). There is sometimes a slight acceleration of alkali-production in the former as compared with that in the deep tubes, but many strains maintain a persistent acid reaction in the flasks for 2 weeks. The difference between tubes and flasks in this respect is not as great as was anticipated.

In other series like that recorded in Table 1, and including all the cultures mentioned in this paper, similar results have been obtained. The strains of *B. paratyphosus* B (22 strains*), *B. enteritidis* (6 strains), and *B. suipestifer* (25 strains) have all begun to show alka-

* One of these strains, 221, has produced alkali much more slowly than the others and shows beginning alkalinity only after about 10 to 14 days. Even then, however, it can be distinguished from all the A strains I have had under observation and in 20 days the difference is still more marked.

linity earlier than any of the 20 strains of *B. paratyphosus* A. The latter without exception have remained more acid than the control tube for, nearly 2 weeks, but in about 14 to 15 days (at 37 C.) many of these approximate the reaction of the control and then become progressively alkaline. The alkalinity of all the A strains at the end of 5 weeks is decided, but is less than that of the others, so that in a rack with 50 to 100 tubes the A milk tubes can be readily distinguished. For the practical differentiation of the A type litmus milk is of the highest value, tho taken by itself the test is probably not absolutely differential any more than any other single test, especially in dealing with old stock cultures that have begun to vary.

INDOL

The nearly statements regarding indol-production by members of this group are conflicting, the result probably of the diversity of methods used.⁸ Since the introduction of the delicate and accurate paradimethyl-amido-benzaldehyde reaction, most observers have agreed that strains giving a positive indol reaction are at least exceedingly rare. I have tested over 200 strains from various sources and have found a positive reaction only in those that show a distinct difference from the true paratyphoid types either biochemically or by agglutination tests.

The indol tests have been made both in the standard peptone medium (paradimethyl-amido-benzaldehyde reaction) and also in most instances by the rapid and convenient modification of Zipfel's tryptophane medium devised by Cannon.⁹ The 74 strains of *B. paratyphosus* A, *B. paratyphosus* B, *B. enteritidis*, and *B. suipestifer* described in this paper have all given consistently negative results. It seems fair to conclude that indol-production by the members of this group is at least as rare as it is among typhoid bacilli.

CARBOHYDRATE-FERMENTATION

The fermentation reactions shown by members of the paratyphoid-enteritidis group have been the subject of extensive study by many investigators, and have constituted the ordinary means of distinguishing

⁸ Kolle and Wassermann, *Handb. d. pathogen. Mikroorganismen*, 1913, 3, p. 1617.

⁹ *Jour. Bacteriol.*, 1916, 1, p. 535.

these organisms from *B. typhosus* and *B. coli*. As well known, the typical carbohydrate reaction (acid- and gas-production) of the three groups is as follows:

TYPE	DEXTROSE	LACTOSE	SACCHAROSE
<i>B. typhosus</i>	—	—	—
<i>B. paratyphosus</i>	+	—	—
<i>B. coli</i>	+	+	±

Only 1 strain among the 74 under observation has failed to conform to this typical classification. This is No. 134, isolated by Dorset in 1899 from the spleen of a pig dying from "acute hog cholera" in Page County, Iowa.¹⁰ This organism resembles the typhoid bacillus in producing acid but no gas in dextrose broth. My own tests, made on this organism from 14 to 16 years after its isolation, show that no change has occurred in this particular. Other characteristics of this bacillus, including some of great diagnostic value, relate it to the paratyphoid-enteritidis group. Similar strains of *B. suis* which do not produce gas in dextrose and other carbohydrate media, but are indistinguishable from the types in other respects, have been isolated by Bock,¹¹ Bainbridge,¹² and others. TenBroeck¹³ has found a non-gas-producing strain in an old stock culture of hog-cholera bacillus that had been under cultivation for 14 years. It resembles the typical hog-cholera bacillus in agglutination and other characters, but fails to form gas in the carbohydrates usually attacked by this organism.

With the exception of No. 134 all the cultures listed agree in their fermentative behavior (gas-production) toward dextrose (+), lactose (—), and saccharose (—).*

The differential diagnosis may be made quite simply by the use of standard nutrient broth tinged with litmus and containing 0.5% of the carbohydrate to be tested. I have also used the Barsiekow media (litmus-nutrose-dextrose and litmus-nutrose-lactose) employed by some German investigators, but so far as my experience goes they afford no additional information in the differentiation of this group.

¹⁰ 18th Ann. Rep., Bur. An. Ind., 1901, p. 566.

¹¹ Arb. a. d. k. Gesndthtsamte, 1906, 24, p. 238.

¹² Jour. Path. and Bacteriol., 1908-9, 13, p. 443.

¹³ Jour. Exper. Med., 1916, 24, p. 213.

* As pointed out by several investigators, certain sources of error in such tests must be guarded against. It is essential to obtain carbohydrates quite free from dextrose. If meat extract or meat infusion is used, it must be free from muscle sugar. When sodium hydrate is used for neutralization, a small bubble of gas may sometimes develop in the culture. Further, unless overheating is avoided in the sterilization of lactose broth, particularly if the reaction is alkaline, a small amount of dextrose will be formed, the presence of which will falsify the fermentation reaction.

A large number of other carbohydrates have been tested by various observers in the study of fermentative characteristics. There is practically general agreement on the results summarized in the following table:

TABLE 3
CARBOHYDRATE-FERMENTATION (ACID- AND GAS-PRODUCTION) BY PARATYPHOID-ENTERITIDIS
BACILLI

+	—	±
Rhamnose Dextrose Galactose Mannose Levulose Maltose* Mannite Sorbitol	Lactose Saccharose Raffinose Dextrin Inulin Erythrite Adonite Salicin	Arabinose Xylose Dulcitol

* Nearly all observers have reported gas- and acid-production in maltose, but May (Jour. Trop. Med. and Hyg., 1911, 14, p. 1) states that only acid was produced in the strain isolated by him from a water supply. Proescher and Roddy (Arch. Int. Med., 1910, 5, p. 263) state that "maltose is not fermented by Para A, but some cultures of Para B cause slight fermentation." In my own series all cultures (except 134) including the para-A strains, have fermented maltose, with gas-production.

There are 3 carbohydrates in this list that are not fermented uniformly by all the members of these groups, namely dulcitol, arabinose, and xylose. It is a disputed question whether such differences in fermentative power are correlated with other characters and whether they permit a differentiation into subgroups of biologic or practical importance.

DULCITOL

Positive dulcitol-fermentation was noted by Voges and Proskauer¹⁴ for certain strains of hog-cholera bacilli and by Conradi, Drigalski, and Jürgens¹⁵ for certain paratyphoid organisms. Kligler¹⁶ has attempted to distinguish between *B. suis* (regarded as dulcitol—) and *B. enteritidis* (dulcitol+) on the basis of dulcitol-fermentation. Ford¹⁷ on the contrary, found that 5 cultures of "the bacillus of hog cholera" agreed in fermenting dulcitol.

The majority of investigators have observed acid- and gas-production in dulcitol by the organisms of this group. This is the case with the strains studied by Morgan,¹⁸ Sacquépé and Chevrel,¹⁹ Boycott,²⁰ MacConkey,²¹ Savage and

¹⁴ Ztschr. f. Hyg. u. Infektionskr., 1898, 28, p. 20.

¹⁵ Ibid., 1903; 42, p. 141.

¹⁶ Jour. Infect. Dis., 1914, 15, p. 187.

¹⁷ Med. News, 1905, 86, p. 1126.

¹⁸ Brit. Med. Jour., 1905, 1, p. 1257.

¹⁹ Ann. de l'Inst. Pasteur, 1906, 20, p. 1.

²⁰ Jour. Hyg., 1906, 6, p. 33.

²¹ Ibid., p. 570.

Gimson,²² Bainbridge,¹² May,²³ Bradley,²⁴ Poppe,²⁵ Seiffert,²⁶ Biewald,²⁷ and Ducamp.²⁸ Morgan, however, records negative results with one strain isolated from hog cholera, and his observation was confirmed for the same culture by Savage,²⁹ who, however, notes that another strain from hog cholera reported by Morgan as giving positive fermentation was negative in his own hands. Ducamp observed that 2 hog cholera strains tested by him did not attack dulcitol. Bradley²⁴ found a difference in the rapidity with which the fermentation is effected: "On dulcitol acid and gas are produced by all the strains (40 stock cultures principally from European laboratories), but the time taken is usually longer than for the other sugars and is especially long with the hog cholera group." Robinson³⁰ has recorded a negative dulcitol reaction for several strains of paratyphoid organisms isolated by him from the feces of 3 persons infected during a water-borne epidemic. One* of these (179 = Robinson's No. 163) has always fermented dulcitol promptly (within 24 hours) since it first came into my hands about 2 years after isolation. When the discrepancy with Robinson's results was noted, the culture was plated and 12 colonies picked. These 12 strains inoculated into dulcitol gave identical reactions, acid and gas in 24 hours. Proescher and Roddy³¹ state that "dulcitol is slightly fermented by 12 per cent. of the cultures"—mostly from human paratyphoid cases—which they examined.

In my own observations the following strains have fermented dulcitol within 24 hours with acid- and gas-production: 2, 5, 8, 12, 47, 130, 149, 150, 151, 152, 179, 180, 185, 202, 203, 209, 210, 211, 221, 223, 225, 62, 115, 161, [169]; the following have failed to ferment dulcitol in 24 hours, but have produced some gas in 5 days (168, 173, 174) (10 days, 172); the following have shown no gas or acid in 15 days: 63, 114, 118, 132, 133, 160, 162, 163, 165, 167, 170, 171, 175, 177, 178.

It thus appears that all 22 of the cultures from human sources ferment dulcitol promptly, while only 4 of 25 of the cultures from swine ferment dulcitol within 24 hours. Five other strains ferment dulcitol tardily (within 15 days). Furthermore, 3 of the porcine cultures giving a prompt positive reaction with dulcitol were received from various laboratory collections under the name of *B. cholerae*-suus, *B. suis*, or *B. anthracis*, and their precise origin is

²² Ibid., 1908, 8, p. 601.

²³ Jour. Trop. Med. and Hyg., 1911, 14, p. 1.

²⁴ Jour. Proc. Roy. Soc. N. S. Wales, 1912, 46, p. 74.

²⁵ Ztschr. f. Infektionskr. d. Haust., 1908-9, 5, p. 42. Quoted by Hübener, Fleischvergiftungen u. Paratyphusinfektionen, Monographie, 1910, p. 78.

²⁶ Ztschr. f. Hyg. u. Infektionskr., 1909, 63, p. 273.

²⁷ Inaug. Diss. Giessen, 1909; cited by Hübener,²⁵ p. 80.

²⁸ These, Lille, 1907; cited by Hübener,²⁵ p. 80.

²⁹ Rep. Med. Officer to Local Gov't Board, 1907-8, p. 425.

³⁰ Jour. Infect. Dis., 1915, 16, p. 448.

* This strain is said to have produced "permanent acidity" in litmus milk, but ever since it came into my hands it has given a typical paratyphus-B reaction (definite alkalinity within 5 days) and has shown no sign of variation within a year tho frequently plated out and tested by transfer of individual colonies.

³¹ Arch. Int. Med., 1910, 5, p. 263.

unknown. Included in this number are 2 cultures from European laboratories: 115 (from Kral) and 161 (from Ostertag). Only one porcine strain (169) whose history is fully known has fermented dulcitate promptly and this was negative when first tested.* The tardy or negative dulcitate-fermentation here manifested is correlated, as will appear, with differences in arabinose-fermentation and agglutination reactions. As already noted, such differences in dulcitate-fermentation as are recorded by previous observers suggest a negative or relatively little dulcitate-fermentation by members of the 'hog-cholera' group (Morgan,¹⁸ Savage,²² Bradley,²⁴ Ducamp,²⁸ Kligler¹⁶).

All 20 strains of *B. paratyphosus*-A type agree in attacking dulcitate tardily. There is no acid or gas formed in any case within 24 hours and in the case of only 3 strains (131, 188, 191) within 48 hours. All but one (11), however, have produced gas and acid in 5 days and this late strain is positive in 10 days. This characteristic of delayed dulcitate-fermentation has not previously been noted as a differential mark. In my series of cultures it distinguishes the *B. paratyphosus*-A strains from the *B. paratyphosus*-B type, all of which (23 strains) ferment dulcitate within 24 hours. In this respect the A strains approximate somewhat to the *B. suispestifer* type, but unlike the latter they all ferment-arabinose promptly—within 24 hours.

All the Gärtner strains (*B. enteritidis*) have fermented dulcitate within 24 hours, in this respect being identical with the *paratyphosus*-B strains.

ARABINOSE

The use of this carbohydrate has been frequently suggested for differential purposes.

Several observers have noted that "hog cholera strains" are particularly apt to give negative results with arabinose.³² Seiffert³⁰ found that several strains of bacilli from paratyphoid fever and meat-poisoning cases and also 2 hog-

* This strain, isolated from the lung of a hog dead from hog cholera, has undergone a definite change since it first came into my hands in June, 1915. It then failed to ferment arabinose in 14 days, and did not attack dulcitate within 48 hours, tho producing both acid and gas within 14 days. Dulcitate-fermentation within 24 hours was still negative on Dec. 2, 1915, April 20, and May 21, 1916 (3 colonies from plates), but on the last named date there was a noticeable acceleration in this respect, and a positive reaction occurred earlier (within 48 hours) than in preceding tests. On Oct. 18, 1916, the culture gave positive dulcitate-fermentation within 24 hours, as did inoculations with 3 colonies picked from an agar plate. On Nov. 10, 1916, 12 colonies picked from a plate gave identical positive results, acid and gas within 24 hours. Coincident with this change in dulcitate-fermenting powers arabinose-fermentation has become positive (acid and gas within 24 hours). Agglutination reactions have been somewhat irregular, but where absorption tests have been made the early ones were of the *suispestifer* type, the later ones of the *paratyphosus*-B type. The 12 strains isolated Nov. 10, 1916, were identical in their agglutination reactions (*paratyphoid*-B¹² serum saturated with *suispestifer* 170) with the typical *paratyphoid*-B strains.

³² Ford, *Med. News*, 1905, 86, p. 1126. Bradley, *Proc. Roy. Soc. N. S. Wales*, 1912, 46, p. 74.

cholera strains (sources not given) fermented arabinose. Langkau³³ observed that 6 strains from calf diarrhea did not ferment arabinose, while others of various origin, including one "Schweinepest" strain, were positive.³⁴ The results reported by others³⁵ do not show complete uniformity; full data are not given in all cases respecting the sources of the cultures tested.

All the strains of the para-B type of human origin in my possession ferment arabinose promptly. On the other hand, only 4 of 25 of the cultures from swine produced gas in arabinose within 24 hours. These are the same strains that ferment dulcitol either slowly or not at all. There is thus an exact correlation in this group of cultures. Those cultures that ferment dulcitol and arabinose promptly are of human origin (23 strains), while the majority of those attacking these carbohydrates tardily or not at all are porcine strains (21 of 25).

XYLOSE

Xylose like arabinose is not fermented uniformly by all strains.

Ford¹⁷ found that some stock "paratyphoid" cultures fermented xylose while others did not. The "hog-cholera" strains that he tested all failed to attack this carbohydrate. Seiffert²⁶ noted a positive result with the strains in his hands as did Biewald.²⁷ Langkau³³ observed that bacilli from calf-diarrhea produced no gas in xylose in the first 24 hours, but could not be distinguished from the other strains after 48 hours. Ducamp²⁸ observed that 2 hog-cholera strains did not attack xylose. Proescher and Roddy³¹ state that "Xylose is fermented by very few cultures of A and B; most of them cause no fermentation." Schern's results have already been mentioned.

An important differential result was obtained by Harding and Ostenberg.³⁰ These investigators, working with fuchsin sulfite media (Endo) found that acid was produced in the presence of xylose by 12 paratyphoid-B and enteritidis strains but not by 1 mouse-typhoid and 5 paratyphoid-A strains. Their results were confirmed and extended by Krumwiede and his coadjutors,³⁷ who found that the 20 para-A strains in their hands failed to ferment xylose while the other members of the paratyphoid-enteritidis group gave positive results. One "hog-cholera" strain, however, fermented only slowly. Apparently only the change in reaction was noted by these investigators, no statement being made as to gas-production.

³³ Inaug. Diss., Leipzig, 1909; cited by Hübener,²⁵ p. 80.

³⁴ Schern's observations (Arb. a.d.k. Gsndtsamte, 1909-10, 33, p. 387) on the behavior of various strains of paratyphoid and hog-cholera bacilli relate to the color changes produced in arabinose and xylose broth tintured with litmus. Altho he notes differences in the strains of human and animal origin, these are not universal, and no attempt is made to correlate them with other characters. It is not apparent from Schern's article whether the differences recorded for various strains—"red," "yellow," "violet," etc.—are constant for each strain under slightly varying conditions of experimentation or over any considerable period of time.

³⁵ Bahr, Raebiger, and Grosso, *Ztschr. f. Infektionskr. d. Haust.*, 1909, 5, p. 295. Bainbridge, *Jour. Path. and Bacteriol.*, 1909, 13, p. 443. Proescher and Roddy, *Arch. Int. Med.*, 1910, 5, p. 263.

³⁶ *Jour. Infect. Dis.*, 1912, 11, p. 109.

³⁷ Krumwiede, Pratt, and Kohn, *Jour. Med. Research*, 1916, 29, p. 355.

In the series with which I have worked, the paratyphoid-A strains are marked off sharply from the others by their inability to ferment xylose. My observations on this point were made for the most part in June, 1915, and are in quite complete accord with those of Harding and Ostenberg and Krumweide, Pratt, and Kohn. None of the paratyphoid-A strains produce either gas or acid in xylose broth. All the other strains here considered produce gas except 134 (atypical suipestifer strain, forming no gas in dextrose) and 153 (*B. enteritidis*). Both these strains, however, produce acid. No. 115 ("Bacillus of hog cholera," Kral's collection) produces acid vigorously, but gas very slowly.

AGGLUTINATION

Rabbits have been used for the agglutination tests. Subcutaneous inoculations of killed bacilli, washed from agar slants, have first been given, followed by intraperitoneal injections of living bacilli. After 4 or 5 intraperitoneal injections the titer of the serum is usually as high as 5000 to 10000, tho there are marked differences in the agglutino-genic qualities of different strains. A high titer—30000 to 40000—has been reached more readily with *B. enteritidis* strains than with the others used for this purpose.

The macroscopic method has been used throughout. All suspensions and dilutions were made with sterile salt solution. Fresh 24-hour-old agar cultures have always been employed. The mixture of serum and bacterial suspensions has been inoculated 2 hours at 37 C., then left overnight, about 18 hours, in the refrigerator and the reading taken. In absorption tests mixtures of serum and absorbing bacilli have been incubated 1 hour at 37 C. before centrifugating.

Four distinct divisions based on agglutinability are recognizable in the organisms here considered: (1) *B. paratyphosus A*, (2) *B. enteritidis*, (3) *B. paratyphosus B*, and (4) *B. suipestifer*.

(1) *B. paratyphosus A*.—Serum produced by organisms of *B. paratyphosus-A* type has agglutinated organisms of the same group to practically the same dilutions as the homologous strains. Table 4 is representative of the results obtained with this serum. All cultures have been tested with this serum, but since the results are similar, it does not seem necessary to tabulate them in detail. Strains 212-219 resemble 191 in showing relatively slight agglutinability in the lower dilutions (1:250), but show a trace of agglutination at 1:5000 as definitely as the homologous strains. As a rule the members of the

other three groups do not show a trace of agglutination at 1:250 or even 1:100 with B.-paratyphosus-A serum. A few anomalous strains, usually stock cultures long under cultivation, exhibit mixed or uncertain agglutinative affinities. These irregular strains will be considered in a later article.

TABLE 4
AGGLUTINATION WITH B.-PARATYPHOSUS-A SERUM (4)

Strain	1:250	1:500	1:1000	1:2000	1:5000	1:10000
3	++	+	+	tr	tr	0
4	++	++	+	+	tr	0
9	+++	++	++	+	0	0
48	++	++	+	+	tr	0
131	++	+	+	+	tr	0
158	+++	++	+	+	tr	0
188	++	++	++	+	tr	0
191	+	+	+	+	tr	0
198	++	++	++	+	0	0
152	0	0	0	0	0	0
B. paratyphosus B						
206	0	0	0	0	0	0
B. enteritidis						
177	0	0	0	0	0	0
B. suipestifer						

+++ = complete
++ = marked
+ = slight
tr = trace
0 = none

(2) *B. enteritidis*.—Serum produced by organisms of *B. enteritidis* type (52) agglutinates the other strains of this group in high dilutions (1:30000), but in only 2 instances has it affected organisms of the suipestifer, paratyphosus-B, or paratyphosus-A groups in a dilution of 1:100 and never in that of 1:250. Conversely the 6 strains of *B. enteritidis* under observation are agglutinated slightly or not at all by highly potent sera of the other groups (Table 5).

TABLE 5

Enteritidis Strain	Suipestifer Serum (118) Titer, 1:5000	Paratyphosus B Serum (12) Titer, 1:10000	Paratyphosus A Serum (4) Titer, 1:3000
50	<100	<250	500
51	<100	<250	100
52	<100	<250	100
53	<100	<250	250
228	<100	<250	100
206	<100	<250	250

(3) *B. paratyphosus B*.—The agglutinating sera produced by various paratyphosus-B strains (2, 5, 12) have given substantially the same results. All have agglutinated the other strains of the

B.-paratyphosus-B group in relatively high dilutions. Many strains of B.-suipestifer are also agglutinated in practically the same dilution by the paratyphosus-B sera, but the paratyphosus-A strains as a rule are not affected by highly potent sera. The B.-enteritidis strains are often slightly agglutinated. One typical series is given in Table 6.

TABLE 6
PARA-B SERUM (5)*

Para B		B. Suipestifer		Para A		B. Enteritidis	
No.	Agglutination	No.	Agglutination	No.	Agglutination	No.	Agglutination
2	5000 +	62	5000 +	3	<100	50	250 +
5	5000 ++	63	1000 +	4	<100	51	250 +
8	5000 +++	114	1000 +	9	<100	52	250 +
12	2000 ++	115	5000 ++	11	<100	53	<250
47	5000 +	118	5000 +	48	<100	228	500 +
		132	250 ++	131	<100		
130	5000 +	133	1000 ++	158	<100		
149	5000 +	134	5000 +++				
150	2000 ++	160	100 +				
151	5000 +	161	500 ++				
152	2000 +	162	500 +				
		163	1000 +				
		165	1000 +				
		167	1000 +				
		168	500 +				
		169	250 ++				
		170	100 +				
		171	1000 +				
		172	100 +				
		173	100 +				
		174	100 +				
		175	500 +				
		177	<100				
		178	250 +				

+++ = complete

++ = marked

+ = slight

* B.typhosus was not agglutinated by this serum at 1:100.

The paratyphosus-B strains are also agglutinated in varying degrees with B.-suipestifer sera. One series is shown in Table 7. Often the agglutination limit is almost as high as the titer of the serum (see especially Nos. 130 and 151). Absorption tests, however, show the real nature of the agglutinative reactions. Table 8, which should be compared with Table 7, affords an excellent demonstration of the group relationship of these organisms. Variations are frequently observed. Thus No. 180 agglutinated feebly or not at all with suipestifer serum for a long time after it came into my possession, but in recent tests it shows greater agglutinability. No. 221 was highly refractory to suipestifer serum at the date of the test recorded in Table 7 (1-19-'17).

TABLE 7
B.-SUIPESTIFER (118) SERUM

Strain	1:250	1:500	1:1000	1:2000	1:5000	1:10000
5	++	+	+	tr	0	0
8	+++	++	+	0	0	0
12	+++	++	+	tr	0	0
47	+++	++	+	0	0	0
130	+++	+++	++	++	+	tr
149	++	++	+	+	0	0
150	++	++	+	+	0	0
151	+++	++	++	++	+	tr
152	tr	0	0	0	0	0
179	++	++	+	+	tr	0
180	++	+	+	tr	0	0
185	++	++	+	tr	0	0
202	++	+	+	tr	0	0
203	+	+	+	tr	0	0
209	+++	++	+	+	0	0
210	++	+	+	tr	0	0
211	tr	tr	tr	tr	0	0
221	0	0	0	0	0	0

+++ = complete
 ++ = marked
 + = slight
 tr = trace
 0 = none

TABLE 8
B.-SUIPESTIFER (118) SERUM SATURATED WITH B. PARATYPHOSUS B (12)

Strain	1:250	1:500	1:1000	1:2000	1:5000	1:10000
5	0	0	0	0	0	0
8	tr	0	0	0	0	0
12	0	0	0	0	0	0
47	tr	0	0	0	0	0
130	tr	0	0	0	0	0
149	0	0	0	0	0	0
150	0	0	0	0	0	0
151	tr	0	0	0	0	0
152	0	0	0	0	0	0
179	tr	0	0	0	0	0
180	0	0	0	0	0	0
185	tr	0	0	0	0	0
202	tr	0	0	0	0	0
203	0	0	0	0	0	0
209	0	0	0	0	0	0
210	0	0	0	0	0	0
211	tr	0	0	0	0	0
221	0	0	0	0	0	0

Table 11 shows the correlation of fermentative and agglutinative characters in the organisms of this group.

(4) *B. suipestifer*.—Agglutinating sera have been produced with various strains of porcine origin (63, 118, 167) and have given relatively uniform results. The majority of the strains of porcine origin agglutinate in as high a dilution as the homologous strains (Table 9). Saturation of the serum with a strain of *B. paratyphosus* B affects but little its agglutimative power for the typical porcine strains (Table 10).

Especial attention may be directed to Nos. 62, 115, and 161, Table 10, which are old stock strains and which, tho presumably of porcine origin, evince an agglutinative affinity to the B.-paratyphosus-B type. These three strains all ferment dulcitol and arabinose promptly and in this respect as in agglutinative reaction resemble the strains of human origin (Table 12). Two strains of porcine origin (Nos. 169 and 175) are not recorded on Tables 9 and 10. These strains, when they were first examined a few months after isolation, showed all the characteristics of what I have called the *suipestifer* type, but in the course of 18 months' cultivation they have altered, both biochemically and agglutinatively, and are now of the paratyphosus-B type in fermentative and agglutinative characters. Sometimes a *suipestifer* serum has been obtained which manifests a definite differential behavior toward the paratyphosus-B and *suipestifer* types without saturation. The action of such a serum is recorded in Table 13. The strains of the paratyphosus-B type (23 of human origin and 5 of porcine origin) are affected slightly or not at all by the *suipestifer* sera.

TABLE 9
B.-SUIPESTIFER (118) SERUM

Strain	1:250	1:500	1:1000	1:2000	1:5000	1:10000
12	++	++	++	+	tr	0
62	+++	++	++	+	0	0
63	+++	+++	+++	++	+	tr
114	+++	+++	++	++	+	tr
115	++	++	+	+	tr	0
118	+++	+++	++	+	tr	tr
132	+++	+++	++	++	+	tr
133	+++	++	++	++	+	tr
160	+++	++	++	++	+	tr
161	++	++	++	++	+	-
162	+++	++	++	++	+	0
163	+++	+++	++	++	+	tr
165	+++	++	++	++	+	tr
167	+++	+++	++	++	+	tr
168	+++	++	++	+	+	tr
170	+++	+++	++	++	++	+
171	++	++	++	++	+	tr
172	+++	++	++	++	+	tr
173	+++	++	++	++	+	tr
174	+++	++	++	++	++	tr
177	+++	+++	+++	++	++	-
178	+++	+++	+++	++	++	tr
	+++	++	++	+	tr	tr

+++ = complete
 ++ = marked
 + = slight
 tr = trace
 0 = none

TABLE 10
B.-SUIPESTIFER (118) SERUM SATURATED WITH B. PARATYPHOSUS B (12)

Strain	1:250	1:500	1:1000	1:2000	1:5000	1:10000
12	tr	0	0	0	0	0
62	+	tr	0	0	0	0
63	++	++	++	+	+	tr
114	++	++	++	+	+	tr
115	tr	0	0	0	0	0
118	++	+	+	+	tr	0
132	++	++	+	+	tr	0
133	++	++	++	+	0	0
160	++	++	++	+	+	tr
161	tr	0	0	0	0	0
162	++	++	++	+	+	tr
163	++	++	+	+	tr	tr
165	++	++	++	+	+	tr
167	++	++	++	+	+	tr
168	++	++	++	++	+	0
170	++	++	++	++	+	0
171	++	++	++	++	+	tr
172	++	++	++	+	+	tr
173	++	++	++	++	+	tr
174	++	++	++	++	+	+
177	++	++	++	++	+	tr
178	++	++	++	++	+	tr

+++ = complete
 ++ = marked
 + = slight
 tr = trace
 0 = none

TABLE 11
STRAINS OF HUMAN ORIGIN

Strain	Rapid Dulcitol- Fermentation (within 24 hr.)	Rapid Arabinose- Fermentation (within 24 hr.)	Agglutination with B.-Paratyphosus-B No. 12 Serum	
			Before Saturation	After Saturation with Suipestifer 167
2	+	+	5000	2000
5	+	+	5000	2000
8	+	+	10000	2000
12	+	+	5000	2000
47	+	+	10000	10000
130	+	+	5000	2000
149	+	+	5000	2000
150	+	+	5000	5000
151	+	+	5000	2000
152	+	+	2000	500
179	+	+	5000	2000
180	+	+	2000	2000
185	+	+	5000	5000
202	+	+	5000	2000
203	+	+	10000	10000
209	+	+	10000	10000
210	+	+	10000	10000
211	+	+	10000	10000
221	+	+	2000	2000
222	+	+	5000	2000
223	+	+	5000	2000
224	+	+	10000	5000
225	+	+	10000	10000

TABLE 12
STRAINS OF PORCINE ORIGIN

Strain	Rapid Dulcitol- Fermentation (within 24 hr.)	Rapid Arabinose- Fermentation (within 24 hr.)	Agglutination with B.-Paratyphosus-B No. 12 Serum	
			Before Saturation	After Saturation with Suipestifer 167
62	+	+	5000	2000
63	—	—	500	<250
114	—	—	500	<250
115	+	+	5000	2000
118	—	—	<250	<250
132	—	—	<250	<250
133	—	—	5000	<250
160	—	—	<250	<250
161	+	+	5000	2000
162	—	—	2000	<250
163	—	—	5000	<250
165	—	—	5000	<250
167	—	—	5000	<250
168	—	—	5000	<250
169	—	—	5000	<250
170	—	—	<250	<250
171	—	—	500	<250
172	—	—	1000	<250
173	—	—	<250	<250
174	—	—	<250	<250
175	—	—	—	<250
177	—	—	1000	<250
178	—	—	1000	<250
234	—	—	2000	<250

TABLE 13
B.-SUIPESTIFER (167) SERUM (TITER, 1:2000)

Strains of Human Origin					Strains of Porcine Origin				
No.	1:250	1:500	1:1000	1:2000	No.	1:250	1:500	1:1000	1:2000
2	+	0	0	0	62	tr	0	0	0
5	0	0	0	0	63	+++	++	+	tr
8	0	0	0	0	114	+++	++	+	tr
12	0	0	0	0	115	tr	0	0	0
47	0	0	0	0	132	+++	++	+	tr
130	+	tr	0	0	133	+++	++	tr	0
149	tr	0	0	0	160	+++	++	+	tr
150	0	0	0	0	161	tr	0	0	0
151	0	0	0	0	162	+++	++	+	tr
152	0	0	0	0	163	+++	++	+	tr
179	tr	0	0	0	165	+++	++	+	tr
180	tr	0	0	0	167	+++	++	+	tr
185	0	0	0	0	168	+++	++	+	tr
202	0	0	0	0	169	0	0	0	0
203	0	0	0	0	170	+++	++	+	tr
209	0	0	0	0	171	+++	++	+	tr
210	0	0	0	0	172	+++	++	+	tr
211	0	0	0	0	173	+++	++	+	tr
221	0	0	0	0	174	+	+	+	+
					175	0	0	0	0
					177	+++	++	+	tr
					178	+++	++	+	tr
					234	+++	++	+	tr

+++ = complete
 ++ = marked
 + = slight
 tr = trace
 0 = none

SUMMARY AND CONCLUSIONS

The cultures of bacilli that I have examined, belonging to the paratyphoid-enteritidis group, fall for the most part into 4 subdivisions.

These may be characterized provisionally as of the following types: (a) *B. paratyphosus* A, (b) *B. paratyphosus* B, (c) *B. suipestifer*, and (d) *B. enteritidis*.

(a) The *B. paratyphosus*-A strains are all from human sources. They attack arabinose quickly and dulcitol slowly; they do not ferment xylose. They produce alkali in litmus milk, but produce it very slowly so that the initial acidity caused by all members of this group persists for a long time and the color of the control tube is rarely regained in less than 2 weeks. A distinct alkaline reaction, however, has been observed in all cultures at the end of 30 days, usually earlier. The strains possessing these characters (20 in number) form a homogeneous agglutinative group.

(b) The *B. paratyphosus*-B strains (23) attack arabinose, dulcitol, and xylose rapidly, generating gas and acid within 24 hours. They produce alkali quickly in litmus milk, so that a definite blue color is observed usually within 4 to 5 days. Although differences occur in the speed of alkali-formation, all the slowest alkali-producing strains of this type that have come under my observation have been in advance of the most rapid alkali-producers of the A type. They are all alike in agglutinative characters. The great majority of the cultures of this type are from human paratyphoid infections. A few cultures of porcine origin also possess these characters.

(c) The *B. suipestifer* strains ferment xylose, but attack arabinose and dulcitol slowly or not at all. None of the typical members of this group produce gas in arabinose or dulcitol within 24 hours, in this respect differing from the *B. paratyphosus*-B strains. They also differ from the latter in their agglutinative characters, though this difference in many cases is manifested only on the application of absorption tests. The *suipestifer* cultures with these characters comprise strains isolated from affected animals in Iowa, Michigan, and Kentucky, as well as stock strains presumably from Maryland and other parts of the United States. In this series the only two European strains said to be of porcine origin (115 from Kral, 161 from Ostertag) possess characters identical with those of the *B. paratyphosus* type. Two strains of known porcine origin (169 and 175) have changed to the *paratyphosus*-B type since first isolated.

(d) The *B. enteritidis* strains are indistinguishable from the *B. paratyphosus*-B strains by any cultural characters, but constitute a distinct agglutinative group.

The strains considered in this paper may be grouped as follows:

Number of Strains	Xylose	Arabinose		Dulcitate		Agglutination Type	Origin	
		Rapid	Slow or Negative	Rapid	Slow or Negative		Human	Porcine
20	—	+			+	Para A	+	
28	+	+		+		Para B	+	(23) + (5)
20	+		+		+	Sulpestifer		+
6	+	+		+		Enteritidis	+	

Division into these four types is based primarily on the examination of recently isolated strains with known histories. Old stock cultures that have been in laboratory collections for some years often show variations, irregularities, and 'departures from type.' Some give reactions, biochemical and agglutinative, that they did not give when first isolated. On the other hand, some strains that apparently possessed unusual fermentative reactions when first isolated later show conformity to type (179).

Variants and irregular strains are also met with which are apparently intermediate between the several groups.

A few strains of porcine origin possess the characteristics of the B.-paratyphosus-B type. These, however, are all strains that have been under cultivation for some time. All the freshly isolated strains from swine that I have been able to obtain, and the majority of the old stock cultures said to be of porcine origin are of the B.-suipestifer type as characterized in the foregoing.

A number of strains, particularly some of the older cultures, have shown marked variation since they came into my hands. The history of these variable and irregular strains will be discussed in a subsequent paper.

Some of the difficulty experienced by previous observers in the differentiation of the B.-paratyphosus-B and B.-suipestifer types has been probably due to the existence of stock cultures labeled in one way or the other but possessing the cultural and agglutinative character of the opposite type. The extent to which transformation of one 'type' into the other occurs under the ordinary conditions of laboratory cultivation is a matter for further investigation.

If the distinction between the freshly isolated cultures of the B.-paratyphosus-B and B.-suipestifer types here indicated is borne out by further studies, it will establish a basis for the tentative classification of this group. No worker with these bacilli, however, can fail to take into account the large amount of variation and the probable transformation of one type into another, especially under the conditions of artificial cultivation.

B. PARATYPHOSUS A

Strain	Labeled	Received from	Date
3	B. paratyphosus.....	Dr. L. O. Scott.....	Feb., 1901
4	B. paratyphosus 16A.....	Amer. Museum Natural History....	Aug., 1912
9	B. paratyphosus 294a.....	Amer. Museum Natural History.....	
11	B. paratyphosus 322A		
(48)	B. paratyphosus a.....	Dr. C. J. Hunt	
131	B. paratyphosus a.....	Dr. Wm. Litterer	
158	B. paratyphosus a.....	Dr. M. M. Canavan.....	
188	Paratyphoid 5.....	Dr. C. L. Cole.....	May, 1916
191	S-n A.....	Dr. C. L. Cole.....	May, 1916
198	Para A.....	Dr. C. L. Cole.....	June, 1916
212	Paratyphosus A H-e.....	Dr. H. Zinsser	Nov., 1916
213	Paratyphosus A E-n.....		
214	Paratyphosus A S-n.....		
215	Paratyphosus A Y-e.....		
216	Paratyphosus A R-n.....		
217	Paratyphosus A B-w.....		
218	Paratyphosus A S-h.....		
219	Paratyphosus A C-l.....		
229	Paratyphosus A No. 8.....	Prof. E. J. McWeeney.....	Dec., 1916
230	Paratyphosus A No. 9.....	Prof. E. J. McWeeney.....	Dec., 1916

B. PARATYPHOSUS B

2	B. paratyphosus 22B.....	Amer. Museum of Natural History.....	
5	B. paratyphosus Buxton.....	Boston Board of Health.....	
8	B. paratyphosus.....	Dr. Theobald Smith.....	1902
12	B. paratyphosus.....		1912
47	B. paratyphosus B.....	Dr. C. J. Hunt.....	1913
130	B. paratyphosus B.....	Dr. Wm. Litterer.....	1914

B. PARATYPHOUS A—Continued

Isolated from	Date	Reference	Remarks
		<i>Jour. Infect. Dis.</i> , 1904, 1, p. 72	
Blood	1907		Received by Amer. Museum of Natural History from Rockefeller Institute, Jan., 1911 Received by Amer. Museum of Natural History from Hygienic Laboratory, Washington
Murray case, paratyphoid epidemic, Boston State Hospital, 1910	Nov., 1914	<i>Boston Med. and Surg. Jour.</i> , 1914, 171, p. 545	
Sergeant K., Band, 28th Infantry, Mission, Tex.	April, 1916		
Blood	May, 1916	Personal communication	
Z., Mission, Texas.....		Personal communication	
Feces.....		<i>Weekly Bull., New York City Health Dept.</i> , Oct. 14, 1916	
			Stock culture, Lister Institute
Stool of paratyphoid case	Aug., 1916	Personal communication	

B. PARATYPHOUS B—Continued

			Stock culture from Rockefeller Institute
			Two other cultures, from other laboratories but similarly labeled, gave identical reactions
Human gallbladder	1899		
Human blood in paratyphoid fever	1912	<i>Irons and Jordan, Jour. Infect. Dis.</i> , 1913, 17, p. 231	Identical with 14 other strains isolated from gallbladder and feces of same patient
Feces; water-borne epidemic of paratyphoid fever	1911	<i>Arch. Int. Med.</i> , 1913, 12, p. 64	
Human blood, paratyphoid fever	1913	Personal communication	Isolated from blood 2 days before case terminated fatally

B. PARATYPHOSUS B—Continued

Strain	Labeled	Received from	Date
149	B. paratyphosus B E3.....	Dr. W. G. Savage.....	1914
150	B. paratyphosus W-s.....	Dr. W. G. Savage.....	1914
151	B. suipestifer Chesterfield.....	Dr. W. G. Savage.....	1914
152	B. suipestifer M-w.....	Dr. W. G. Savage.....	1914
179	B. paratyphosus Put-In-Bay.....	Dr. G. H. Robinson.....	Nov., 1915
180	B. paratyphosus W-y.....	Dr. H. S. Bernstein.....	Feb., 1916
185	B. paratyphosus B-y.....	Dr. C. L. Cole.....	May, 1916
202	B. paratyphosus B VIII.....	Dr. J. G. Cumming.....	June, 1916
203	B. paratyphosus B IX.....	Dr. J. G. Cumming.....	June, 1916
209	B. paratyphosus B.....	Major Siler, U. S. A.	Oct., 1916
210	B. paratyphosus B.....	Major Siler, U. S. A.	Oct., 1916
211	B. paratyphosus B.....	Major Siler, U. S. A.	Oct., 1916
221	B. paratyphosus B 338.....	Dr. A. B. Wadsworth... ..	Nov. 1916
222	"B. Aertryke No. 1, Strain H"....	Prof. E. J. McWeeney.....	Dec., 1916
223	B. Aertryke No. 2.....	Prof. E. J. McWeeney.....	Dec., 1916
224	B. paratyphosus B, No. 3, Strain R d	Prof. E. J. McWeeney.....	Dec., 1916
225	B. paratyphosus B, No. 4.....	Prof. E. J. McWeeney.....	Dec., 1916

B. SUIPESTIFER

62	B. cholera-suis III.....	Johns Hopkins Pathological Lab- oratory	Oct., 1902
63	B. cholera-suis IV "1809 Maryland"	Dr. Theobald Smith.....	Oct., 1902
114	Bacillus of hog cholera No. 649....	Dr. W. E. King.....	April, 1914

B. PARATYPHOSUS B—Continued

Isolated from	Date	Reference	Remarks
Feces of case of paratyphoid fever	1908	Rep. of Med. Officer to Local Govt. Bd., 1908-9, p. 316. Strain (E) Case 1, p. 324	
Feces of case of paratyphoid fever (fatal)	1908	Like 149, strain (w) Case 4, p. 325	
Food poisoning cases, Chesterfield, England	1911	Peck and Thomson, Special Report on Outbreak of Food Poisoning in Chesterfield, 1911	Obtained by Dr. Savage direct from Dr. Thomson
Organs of fatal food poisoning cases	1908	Savage and Gunnison, Jour. Hyg., 1908, 8, p. 601	Savage regards this organism as a sulpestifer: "the brawn was certainly infected before cooking"
Feces, Put-In-Bay epidemic	1913	Jour. Infect. Dis., 1915, 16, p. 448	
Pie mixture, food poisoning outbreak, Westerly, R. I.	1915	Jour. Amer. Med. Assn., 1916, 66, p. 167	
Lymph-gland infection in man		Jour. Infect. Dis., 1916, 18, p. 349	
Human gallbladder at autopsy	Oct., 1915	Personal communication	"Almost pure culture in gallbladder"
Human case of paratyphoid in San Francisco	Dec., 1914	Personal communication	
Blood, case of paratyphoid in San Antonio, Texas	July, 1916	Personal communication	
Blood, case of paratyphoid in San Antonio, Texas	Aug., 1916	Personal communication	
Feces, case of paratyphoid in San Antonio, Texas	Aug., 1916	Personal communication	
Feces of paratyphoid-carrier	April, 1916	Monthly Bull. N.Y. State Dept. of Health, Oct., 1916 p. 252	
Intestine of fatal case of gastro-enteritis, possibly due to food-poisoning	Sept., 1915	Brit. Med. Jour., Sept. 30, 1916	
Stock culture from Lister Institute, London			Obtained by Prof. McWeeney from Lister Institute "some years ago"
Fatal case of paratyphoid fever	1908	Jour. Hyg., 1911, 11, p. 68	
Feces, case of paratyphoid	1916	Personal communication	

B. SUIPESTIFER—Continued

			Obtained by Johns Hopkins "from the laboratory of the Bureau of Animal Industry, Washington, D. C. Since 1897 it has been in our own stock and perhaps some few years longer"
		Smith and Reagh, Jour. Med. Research, 1903, 4, p. 272	Regarded as a typical hog-cholera bacillus (Hog cholera a, T. Smith)
			Received originally from Dr. T. Smith. "Passed several times through hogs"

B. SUIPESTIFER—Continued

Strain	Labeled	Received from	Date
115	Bacillus of hog cholera No. 050....	Dr. W. E. King.....	April, 1914
118	Bacillus of hog cholera No. 053....	Dr. W. E. King.....	April, 1914
132	B. cholera-suis B.....	Dr. Marion Dorset.....	May, 1914
133	B. cholera-suis C.....	Dr. Marion Dorset.....	May, 1914
134	B. cholera-suis.....	Dr. Marion Dorset.....	May, 1914
160	B. cholera-suis No. 17008.....	Dr. C. W. Brown.....	June, 1915
161	B. cholera-suis No. 17003.....	Dr. C. W. Brown.....	June, 1915
162	B. cholera-suis No. 17009.....	Dr. C. W. Brown.....	June, 1915
163	B. cholera-suis No. 17001.....	Dr. C. W. Brown.....	June, 1915
165	B. suipestifer A.....	Dr. D. J. Healy.....	July, 1915
167	B. suipestifer 1.....	Dr. R. E. Buchanan.....	June, 1915
168	B. suipestifer 3.....	Dr. R. E. Buchanan.....	June, 1915
169	B. suipestifer 7.....	Dr. R. E. Buchanan.....	June, 1915
170	B. suipestifer 8.....	Dr. R. E. Buchanan.....	June, 1915
171	B. suipestifer 14.....	Dr. R. E. Buchanan.....	June, 1915
172	B. suipestifer 18.....	Dr. R. E. Buchanan.....	June, 1915
173	B. suipestifer 27.....	Dr. R. E. Buchanan.....	June, 1915
174	B. suipestifer 33.....	Dr. R. E. Buchanan.....	June, 1915
175	B. suipestifer 39.....	Dr. R. E. Buchanan.....	June, 1915
177	B. suipestifer 51.....	Dr. R. E. Buchanan.....	June, 1915
178	B. suipestifer 55.....	Dr. R. E. Buchanan.....	June, 1915
234	B. suipestifer.....	Prof. D. J. Healy.....	Jan., 1917

B. ENTERITIDIS

50	B. enteritidis Gärtner 18.....	Amer. Museum Natural History....	1913
51	B. enteritidis Gärtner.....	University of Chicago.....	
52	B. enteritidis II Gärtner.....	Johns Hopkins Pathological Lab oratory	Oct., 1902
53	B. enteritidis.....	Dr. C. J. Hunt.....	Oct., 1913
206	B. enteritidis P-r VI.....	Dr. J. G. Cumming.....	June, 1916
228	B. enteritidis Limerick.....	Prof. E. J. McWeeney.....	Dec., 1916

B. SUIPESTIFER—Continued

Isolated from	Date	Reference	Remarks
			Received originally from Kral's collection
Spleen of hog.....	Jan., 1903		Isolated by Dr. Boxmeyer
Hog inoculated with hog-cholera virus	Jan., 1908		Isolated from hog inoculated with "Hemstock virus," a strain of hog-cholera virus obtained from a typical outbreak of hog cholera in Iowa Virus known as "Herd 1 disease"
Guinea-pig inoculated with hog-cholera virus	1904	Bull. 72, Bur. An. Ind., pp. 6, 43	
Spleen of a pig dying from "acute hog-cholera"	1899	18th Ann. Rep., Bur. An. Ind., 1901, p. 566	
Hog, epidemic of hog cholera in Michigan	Before 1913		Nos. 160, 162, and 163 were isolated from affected animals in epidemics of hog cholera in different localities in Michigan
			Received originally from Dr. Ostertag
Hog, epidemic of hog cholera in Michigan	Before 1913		See remarks on 160
Hog, epidemic of hog cholera in Michigan	Before 1913		See remarks on 160
Spleen of hog acutely ill with hog cholera	Aug., 1914		
Spleen, hog	1915		
Spleen, hog			
Lung, hog			
Spleen, hog			
Lung, hog	"Spring" of 1915		167-175 inclusive: From hogs slaughtered for virus at serum plant, Ames, Iowa. All showed typical postmortem lesions of hog cholera
Spleen, hog			
Lung, hog			
Spleen, hog			
Lung, hog	1915		
Lung, hog			
Intestine, hog			
Spleen, hog	Nov., 1916		

B. ENTERITIDIS—Continued

			Originally from the collection of Rockefeller Institute
			Source unknown. Stock culture
			"From the laboratory of Dr. Herbert Durham, University of Cambridge, England, in 1900. It is probably much older"
Feces: "Epidemic of paratyphoid B infection"	Dec., 1911	Personal communication	"Agglutinins in sera for this organism and for B. paratyphoid B"
Stool of man who was attending the calves during epidemic of enteritidis	Dec., 1914		"Agglutinates 1-10,000. Typical sugar reactions"
Organs of a fatal case of food-poisoning	1908	Brit. Med. Jour., 1909, 1, p. 1171	

THE OXIDASE OF RHUS DIVERSILOBA*

JAMES B. MCNAIR

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THE PRESENCE OF THE OXIDASE IN THE PLANT AND ITS RELATION TO THE PLANT POISON

It has often been noticed that when a plant of *Rhus diversiloba*, T. and G., is injured, the exuding sap rapidly becomes brown and hardens into a black, shiny, varnish-like substance. This phenomenon becomes more interesting when it is realized that the violently irritating properties of the fresh sap are completely lost during the formation of this resin-like body. This change in toxic properties may be readily followed in connection with the darkening of the sap, as the poisonous property of the freshly exuded sap is soluble in 95 per cent. alcohol, as well as its nontoxic black oxidation product. The poisonous and nonpoisonous alcohol-soluble substances may readily be separated, however, for the oxidation product is insoluble in xylol, whereas the toxic substance is soluble in xylol.

No evidence is here set forth to prove that the substance darkened is the poison. The darkening of the sap through oxidation is considered here merely as an indicator of the extent of the loss of toxic properties.

This darkening and loss of toxic properties seems to be mainly due to oxidation in the presence of moisture, as the following experiments indicate:

Sap from a fresh incision in a rhus plant was thinly coated for about 1 sq. cm. on the inside of test tubes, $\frac{3}{4}$ by 6 inches in size, which had been thoroughly cleaned in concentrated sulphuric acid saturated with potassium dichromate. The tubes were now filled with gases as follows: 2 with oxygen; 2 with hydrogen from the reaction of zinc and sulphuric acid, purified by bubbling successively through 10% lead nitrate, 10% silver nitrate, and alkaline pyrogallol; 2 with carbon dioxide; 2 with nitrogen secured by shaking air with alkaline pyrogallol in a Hempel pipet; and 2 with air. These gases, before entrance to the test tubes, were dried by passing through calcium-chlorid tubes. One test tube of each gas had 1 c.c. of distilled water placed in it. The tubes were finally corked with rubber corks, sealed with paraffin, and kept at the

* Received for publication December 15, 1917.

temperature of the surrounding air, which at this time ranged between 13 and 19 C. The results are as follows:

- In dry air, the sample did not appreciably darken.
- In moist air, the sample darkened after 5 hours.
- In dry oxygen, the sample did not appreciably darken.
- In moist oxygen, the sample darkened after 3 hours.
- In dry hydrogen, the sample did not appreciably darken.
- In wet hydrogen, the sample darkened after 2 days.
- In dry carbon dioxid, the sample did not appreciably darken.
- In wet carbon dioxid, the sample darkened very slowly after 2 days.
- In dry nitrogen, the sample did not appreciably darken.
- In wet nitrogen, the sample darkened after 2 days.
- In 20 mm. vacuo, the sample darkened after 2 days.



Fig. 1. *Rhus-diversiloba* leaf. A and B indicate the resinified poison on insect-injured leaf.

The absorption of oxygen during the drying of the sap can be readily followed by its increase in weight.

Fresh sap spread in a thin layer within a beaker was dried in a desiccator in an atmosphere of hydrogen until constant weight had been attained (about 12 hours). The sap-coated beaker thus freed from moisture was placed in a damp place for several days. At the expiration of this time the beaker and contents were again placed in a desiccator until constant weight was secured. An increase in weight of approximately 1%, together with the hardening and darkening of the sap, had occurred. The increase in weight, however, did not cease after several days, but continued for at least a month.

By the use of a combustion furnace it was found that fresh sap when dried contains a lower percentage of oxygen than a specimen which has been exposed to the air for a week. All the other elements

remain constant. The increase in weight, therefore, is due to oxidation.

Experiments were next conducted to determine the effect of different temperatures upon the darkening of the expressed sap.

Test tubes, the same size as those described and similarly cleaned, were thinly coated inside, over about 1 sq. cm., with fresh sap. One cubic centimeter of distilled water was now placed in each test tube, and rubber corks introduced. They were then individually subjected to the following temperatures:

- At 5 C. a sample did not appreciably darken.
- At 20 C. a sample darkened after 4 hours.
- At 30 C. a sample darkened after 4½ hours.
- At 40 C. a sample darkened after 5 hours.
- At 50 C. a sample darkened after 7 hours.
- At 60 C. a sample darkened after 24 hours.
- At 80 C. a sample did not appreciably darken.
- At 100 C. a sample did not appreciably darken.

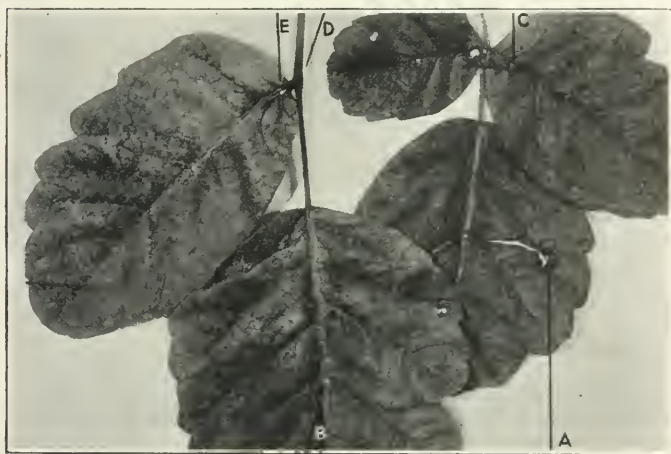


Fig. 2. *Rhus-diversiloba* leaves. A indicates resinified poison on injured part; D, resinified poison at base of leaflet; B, C, E, scale insects (*Aspidiotis*). A case of natural immunity. The scale insects were identified by Mr. G. A. Coleman.

These experiments indicate that the optimum temperature for the oxidation of the sap by the enzyme lies somewhere between 5 and 30 C. and nearer 20 than 30 C. The total range of its activity lies between 5 and 80 C.

Atkins¹ has observed that a browning of expressed sap is invariably associated with the presence of an oxidase. (The brown saps which Atkins investigated were those from: *Hedera helix*, *Syringa vulgaris*,

¹ Sc. Proc. Roy. Dublin Soc., 1913, 14, p. 144.

Magnolia acuminata, *Catalpa bignonioides*, *Fraxinus oxyphylla*, *Fraxinus excelsior*, and *Helianthus multiflorus*). This phenomenon is analogous to the changing of the sap of the lactree, *Rhus vernicifera*, when exposed to the air for some time. It is transformed into a very beautiful black varnish, which the Chinese and Japanese use for varnishing their furniture.² It is not surprising then that an oxidase should be found in *Rhus diversiloba*.

Similar oxidizing enzymes have been found by Bertrand³ in plants destitute of chlorophyl; for example, 2 mushrooms, *Boletus euridus* and *Agaricus sanguineus*. The enzyme, then, is not necessarily a dependent of chlorophyl, and when present in green plants it might be expected to continue its work of oxidation in the leaves after they have become destitute of chlorophyl. The death of the protoplasm, as well as the presence of conditions unfavorable to the normal metabolism of the cell, might not improbably be followed by increased oxidase activity. This has been found true in the case of insect injury to *Rhus diversiloba*, abscission of the mid-rib in the leaf, severe twisting of the stem, and a number of other instances. It has been noticed when mulberry trees were cut back too frequently that an abnormal yellow color and crinkled appearance resulted in the leaves; Suzuki⁴ investigated this to find that an excessive production of oxidases had taken place in such yellow areas. This he attributed to the lack of proper nutrition of rapidly growing tissues. Woods⁵ observed a similar phenomenon in the 'mosaic disease' of tobacco plants which had been cut back.

Bunzel⁶ investigated the oxidase content of the normal and 'curly top' diseased leaves of a sugar beet. He found that the diseased leaves had an oxidase content 2 or 3 times greater than that of healthy and normally developed ones. The roots of the two kinds of plants showed no marked differences however.

When the mature leaves of *Rhus diversiloba* are green, they give no oxidase reaction with gum guaiac.

For this experiment a finely chopped leaf was placed in a test tube with 10 c.c. of water heated to 25 C. Enough alcoholic solution of guaiac (strength about 1:60) to cause a turbidity was added. No blue color appeared in this mixture before it did in an aqueous check solution of the same temperature and concentration of guaiac. The failure of the green leaves to give this oxidase reaction may have been due to the presence of tannin or gallic acid

² Yoshida, Jour. Chem. Soc. Transactions, 1883, 43, p. 472. Yoshida and Korschet, As. Soc. Japan, 12, p. 182.

³ Compt. rend. Acad. d. sci., 1894, 118, p. 1215. Bull. Soc. chim., Paris, 1894, 11, p. 717. Compt. rend. Acad. d. sci., 1895, 120, p. 266; 121, p. 166; 1896, 122, p. 1132. Bull. Soc. chim., Paris, 1896, 15, p. 791. Compt. rend. Acad. d. sci., 1896, 122, p. 1215. Bull. Soc. chim., Paris, 1896, 15, p. 793. Jour. Pharm. et Chim., 1896, 3, p. 607. Compt. rend. Acad. d. sci., 1896, 123, p. 463. Ann. Chim. et Physique, 1897, 12, p. 115. Bull. Soc. chim., Paris, 1897, 17, p. 753. Compt. rend. Acad. d. sci., 1897, 124, pp. 1032, 1355. Jour. Pharm. et Chim., 1897, 5, p. 545. Arch. Agronom., 1897, 23, p. 285. Bull. Soc. chim., Paris, 1897, 17, p. 577. Ann. de l'Inst. Pasteur, 1902, 16, p. 179. Compt. rend. Acad. d. sci., 1907, 145, p. 340. Bull. Soc. chim., Paris, 1908, 145, p. 1352. Compt. rend. Soc. de biol., 1896, 48, p. 811; 1895, 47, p. 579.

⁴ Bull. Agr. College Tokyo, 1900, 4, p. 167.

⁵ Woods, U. S. Dept. Agr. Bull., 18, 1902.

⁶ U. S. Dept. Agr., Bur. Plant Ind. Bull., 277, 1913.

in them, as these substances have been shown by Hunger⁷ to interfere with the guaiac test. The presence of tannin, gallic acid, or gallotannin was indicated by the reagent of Fletcher and Allen⁸ (0.05 gm. of pure potassium ferricyanid dissolved in 50 c.c. of water, and an equal volume of concentrated ammonia water added). This indicator produces a red coloration with tannin, gallic acid, or gallotannic acid in solution, being so sensitive that a drop of the indicator will detect 1 part of tannin in 10,000 parts of water.

A positive test for oxidase may be secured with guaiac after the inhibitor has been removed. A rapid method for separating the inhibiting substance and enzyme is that employed by Aso⁹ for tannin. The precipitation of the enzyme may be effected by pouring the expressed sap into 6 volumes of 95% alcohol. The precipitate when redissolved in water gives the guaiac reaction. This color is destroyed by addition of the filtrate.

In autumn when the leaves become red and yellow, positive results are obtained for oxidase with gum guaiac and the leaves contain no tannin. (If sufficient tannin was added to the solution before testing, the guaiac test became negative.)

The alcoholic solution made from the autumnal leaves is less toxic than a similar solution of equal concentration from the normal leaves when applied to histologically corresponding areas of skin of a very sensitive person. Of the autumnal leaves the red are less toxic than the yellow, and when the leaves have finally withered and fallen they are nontoxic. I am indebted to Dr. Edward von Adelung, who is very sensitive to the poison, for the determination of these differences in toxicity.

This gradual loss of toxicity of the leaves through oxidation has led several observers to consider the poison volatile. For instance, Mackie,¹⁰ writing on the value of oak leaves for forage, says: "It would seem that the irritating and poisonous oil of poison oak is volatile at a comparatively low temperature. In gathering the specimens the writer was badly poisoned even though gloves were worn; yet after drying at ordinary room temperature, and the leaves pressed into the mill with bare hands, no poisoning effects followed." That there is no volatile poison in the plant was known to Dr. von Adelung,¹¹ and this has been verified by further experiments in a more recent article.¹² This loss of toxicity is therefore due to the oxidation of the poison and the loss of fluidity of the sap.

⁷ Ber. die Bot. Ges., 1901, 19, p. 648.

⁸ Chemical News, 29, pp. 167 and 189.

⁹ Botanical Magazine, Tokyo, 1890, 14, No. 166.

¹⁰ California Exper. Sta., Bull. 150, 1903.

¹¹ Arch. Int. Med., 1913, 2, p. 184.

¹² McNair, Jour. Infect. Dis., 1916, 19, p. 429.

THE PREPARATION OF THE ENZYME EXTRACT

The plant leaves were used as sources of the ferment and the following method was pursued in its preparation: Fresh green leaves were washed in many changes of water, finally in distilled water. After draining, the leaves were finely cut with a meat chopper. The finely chopped material was mixed with distilled water, glycerin, and a small amount of chloroform, and allowed to soak for 48 hours. The mixture was now filtered through cotton and the filtrate reserved. (As this solution was made from green leaves it contained tannin but not sufficient to inhibit the guaiac reaction. In fact for some reason not investigated the addition of tannin to this solution did not interfere with the guaiac test.)

The constants of the filtered enzyme solution were as follows:

Density—23 degrees Baume (sp. gr. 1.190) at 18.5 C.
 Glycerol content—73%, or 270.37 parts by weight of glycerin
 for each 100 parts of water.
 Ash—1.666 gm. per liter, or 0.0016%.*

* Tannin, 0.0012 per cent, estimated by a comparison of the amount of ash with the amount of ash and tannin obtained by Mackie¹⁰ from water-free *Rhus-diversiloba* leaves. His analysis yielded 9.15 per cent. ash, 7.24 per cent. protein, 26.56 per cent. fiber, 6.79 per cent. tannin (Günther), 43.39 per cent. nitrogen-free extract, and 6.87 per cent. ether extract.

When tested according to the "Qualitative Analysis" of Morgan, 1907, p. 239, the ash gave positive indications for iron, aluminum, calcium, magnesium, silica, sodium, potassium, and phosphorus.

This ash analysis shows the oxidase to be unlike that of Bertrand obtained from *Rhus vernicifera*, which contained manganese. On the other hand, Sarthou¹³ found the oxidase of *Schinus molle* (a tree of the same family as *Rhus diversiloba*, namely, the *anacardiaceae*) to contain iron, calcium, sodium, but no manganese.

This enzyme solution was used in the experiments which follow.

RELATION OF THE MANNER OF ACTION OF THE ENZYME TO THE
CHEMICAL COMPOSITION OF THE POISON

Emil Fisher¹⁴ has evolved the hypothesis that in the majority of ferment actions there exists a stereochemic relation between the acting substance and the body acted upon. According to him, it is necessary that the ferments and the substances they act upon, have like geometric structures.

Any clue as to the nature of the substances acted upon by the oxidase of *Rhus diversiloba* may therefore be expected to throw some light upon the nature of the poison itself. So far all that has been done in the direction of defining the nature of this enzyme has been the discovery of its action upon the poison and the circumstances of reaction, etc., favoring this action. The oxidation of the poison by its enzyme is favored by alkalis — for example, potassium hydroxid and sodium hydroxid — and may be greatly retarded, if not inhibited, by the presence of acids.

¹³ Jour. Pharm. et Chim., 1900, 11, p. 482; 12, p. 583; 1901, 13, p. 464.

¹⁴ Ber. d. deutsch. Chem. Ges., 1894, pp. 2071, 2985, 1429, 3499.

Enzymes are considered as catalyzers and merely accelerate an oxidation that would take place in their absence at a slower rate. Attention was therefore directed to readily oxidizable substances. Of bodies most readily oxidized those of the aromatic series seem to be more susceptible than those of the aliphatic series. As the oxidation of polyphenols is considerably increased by small quantities of alkali,¹⁵ it was thought the enzyme might be a phenolase. Consequently its action was tested with the diphenols, hydroquinon, pyrocatechin, resorcin, and the triphenols, phloroglucin and pyrogallol.

The Preparation of Hydrochinon Solution and the Colorimetric Method of Testing.—One gram of hydrochinon was weighed rapidly so as to prevent oxidation as much as possible. The weighed phenol was placed in a 250-c.c. Erlenmeyer flask together with 99 c.c. of distilled water. Three such solutions were made with hydrochinon. From 5 to 15 c.c. of the enzyme solution were added to individual phenol solutions.

Some of the enzyme solution was boiled for 5 minutes (distilled water being added to keep the volume constant). After boiling and consequent cooling to room temperature this destroyed enzyme solution was added in varying amounts to 1% hydrochinon solutions. The three phenol solutions, one normal, another with the added enzyme, and the third with the destroyed enzyme, were subjected to a temperature varying from 14 to 15 C. for 3.5 hours.

As hydrochinon has a strong tendency to oxidize in the air without any accelerating enzyme, check solutions were used in which equal amounts of the substance tested were placed without the enzyme. In order to decide whether the reactions were due to enzymatic or to an ordinary chemical change caused by the glycerin plant extract, checks were used in which an equivalent amount of enzyme solution was boiled before being added to the phenol solution. As the reaction was not equally accelerated by the boiled enzyme, it was concluded that its diminution in activity was caused by the destruction of a thermolabile oxidizing agent. The results are recorded in Table 1.

TABLE 1
FINAL COLORS AS ESTIMATED BY A DUBOSCQ COLORIMETER

	No Enzyme (Depth, mm.)	5 c.c. Enzyme Solution (Depth, mm.)	10 c.c. Enzyme Solution (Depth, mm.)	15 c.c. Enzyme Solution (Depth, mm.)
Normal enzyme.....	20	23.5	26.5	31.0
Boiled enzyme.....	20	21.0	22.5	25.0
Difference in depth of color....	0	2.5	4.0	6.0

The results obtained in a series of experiments were not very satisfactory and therefore the Van Slyke apparatus was tried.

The Method of Testing the Oxidase with the Van Slyke Apparatus.—The apparatus used was that described by Van Slyke.¹⁶ The structure of the

¹⁵ Coleman and Walker, Organic Chemistry, p. 409.

¹⁶ Jour. Biol. Chem., 1912, 12, p. 275.

apparatus and the manner in which it was set up are apparent from the accompanying cut and photograph.

As the Van Slyke apparatus does not appear to have been hitherto employed for the study of oxidases, directions for its manipulation follow:

1. The admission of the aromatic compound: Water from F fills the capillary leading to the Hempel pipet and also the other capillary as far as C. Into A one pours a volume of the phenolic solution to be oxidized sufficient

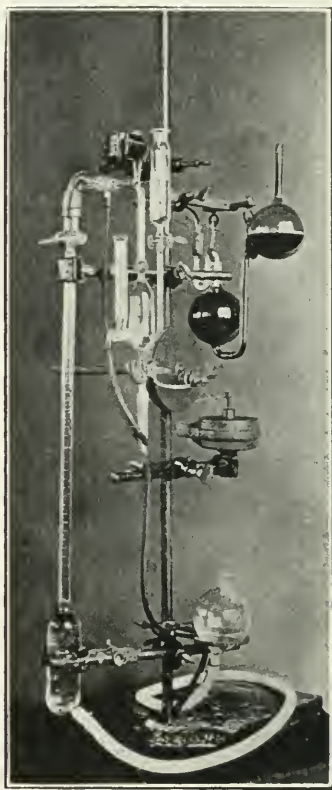


Fig. 3. The complete Van Slyke apparatus.

to fill D up to the 20-c.c. mark (the excess may be drawn off by the cock d). One then closes a and turns c and f so that D and F are connected.

2. The admission of the enzyme solution and the oxidation of the aromatic compound: One places the enzyme solution in B. The admission of the desired amount of enzyme solution into the oxidizing bulb D is accomplished by opening the cock between B and D. The oxidizing bulb is now connected with the motor, as shown in the photograph, and shaken for the determined time.

3. The determination of the amount of oxygen absorbed by the aromatic compound: The reaction being completed, all the gas in D is displaced into F by distilled water from A and a buret reading taken by closing the cock f and lifting the bulb attached to the buret, until the meniscus in the buret and that in the bulb are on the same horizontal plane. After the reading is taken, the gas is forced over from F into the absorption pipet by merely opening f and lifting the buret bulb. The driving rod is then connected with the pipet by lifting the hook from the shoulder of d and placing the other hook, on the opposite side of the driving rod, over the horizontal lower tube of the

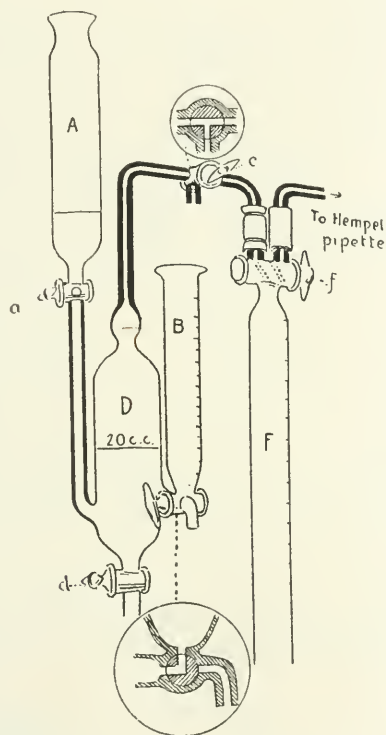
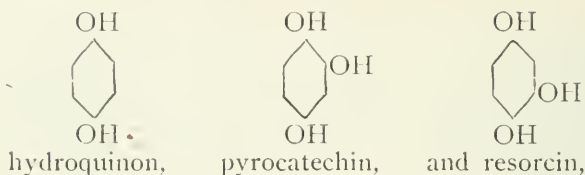


Fig. 4. Oxidizing bulb and connections in detail. The Van Slyke apparatus.

pipet. The latter is then shaken by the motor for 5 minutes, which, with any but almost completely exhausted alkaline pyrogallol solutions, completes the absorption of the residual oxygen. The residual, gas mostly, nitrogen is then measured in F.

The alkaline solution of pyrogallol used in the Hempel pipet, is prepared by mixing together directly in the absorption pipet 5 gm. of pyrogallol dissolved in 15 c.c. of water, and 120 gm. of potassium hydroxid dissolved in 80 c.c. of water.

When tried with 3 isomeric diphenols, namely,



in the Van Slyke apparatus, it was seen that the quantity of gas absorbed was almost nil for the metadiphenol (resorcin), while the paradiphenol (hydroquinon) oxidized very strongly. The ortho diphenol (pyrocatechin) maintained an intermediate position.

TABLE 2
RESULTS OF THE TEST OF THE ENZYME WITH THE VAN SLYKE APPARATUS

2% hydroxy- phenol (c.c.)	Enzyme Solution (c.c.)	Oxidizer Shaken (min.)	Gas (c.c.)	Pipet Shaken (min.)	Gas (c.c.)	Difference in Amount of Gas (c.c.)	Accelera- tion
Hydrochinon							
20	2	10	34.7	5	29.6	5.1	0.8
20	0	10	34.5	5	28.6	5.9	
Pyrocatechin							
20	2	10	35.4	5	29.9	5.5	0.6
20	0	10	34.1	5	28.0	6.1	
Resorcin							
20	2	10	34.8	5	28.7	6.1	0.1
20	0	10	34.4	5	28.2	6.2	

Room temperature throughout the experiment 23.5-24 C.
Barometric pressure throughout the experiment 752 mm.

The action of the enzyme was tested on 2 isomeric triphenols, namely, phloroglucin, in which all the hydroxyls are in the meta position (symetrically placed), and pyrogallol, with its consecutive hydroxyls. It was found that phloroglucin did not oxidize, while pyrogallol absorbed oxygen rapidly.

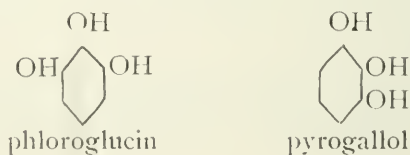


TABLE 3
 RESULTS OF TEST OF THE ENZYME WITH PHLOROGLUCIN AND PYROGALLOL

2% hydroxy- phenol (c.c.)	Enzyme Solution (c.c.)	Oxidizer Shaken (min.)	Gas (c.c.)	Pipet Shaken (min.)	Gas (c.c.)	Difference in Amount of Gas (c.c.)	Accelera- tion
Pyrogallol							
20	2	10	34.1	5	28.8	5.3	0.3
20	0	10	34.1	5	28.5	5.6	
Phloroglucin							
20	2	10	34.5	5	28.3	6.2	0.0
20	0	10	34.8	5	28.6	6.2	

Room temperature throughout the experiment 23.5-24 C.

Barometric pressure throughout the experiment 752 mm.

From the foregoing it would seem that the enzyme is a soluble ferment accelerating the oxidation of bodies of the phenol series, which possess at least 2 hydroxyl groups, when these groups occupy the para or ortho position.

The experiments recorded in Table 4, in which the amount of substrate is constant, show that this enzyme obeys the rule expressed by the formula $F.t = \text{const.}$ In other words, the acceleration of oxidation is directly proportional to the concentration of the ferment, and the time required to achieve a given amount of oxidation is inversely proportional to the amount of ferment present.¹⁷

 TABLE 4
 RESULTS OF THE TEST OF THE ENZYME WITH THE VAN SLYKE APPARATUS

2% Hydro- chinon (c.c.)	Enzyme Solution (c.c.)	Oxidizer Shaken (min.)	Gas (c.c.)	Pipet Shaken (min.)	Gas (c.c.)	Difference between Amounts of Gas (c.c.) (observed equivalent constant)	Constant Calculated $ft = k$ $k = 30$
20	1	30.0	34.4	5	28.4	6.0	30
20	2	15.0	34.3	5	28.8	5.5	30
20	3	10.0	34.9	5	28.6	6.3	30
20	4	7.5	35.1	5	28.6	6.5	30
20	5	6.0	35.6	5	29.1	6.5	30
	f	t				k	k

Room temperature throughout experiment 18 C.

Barometric pressure throughout experiment 759 mm.

¹⁷ Hedin, Jour. Physiol., 1905, 32, p. 468; 1906, 34, p. 370. Hoppe, Seyler's Ztschr. f. physiol. Chem., 1908, 57, p. 471.

The enzyme solution was found to accelerate the oxidation of other bodies of the aromatic series, for example those containing amin groups, as paramidophenol $C_6H_4.OH.NH_2(1,4)$ and paraphenylenediamin $C_6H_4.NH_2NH_2(1,4)$.

TABLE 5
DEPENDENCE OF RAPIDITY OF OXIDATION UPON THE CONCENTRATION OF ENZYME

2% Hydrochinon (c.c.)	Enzyme Solution (c.c.)	Oxidizer Shaken (min.)	Gas (c.c.)	Pipet Shaken (min.)	Gas (c.c.)	Difference between Amounts of Gas (c.c.) (4-6)	Comparative Amount of Absorption (7-n)	Calculated*
20	0	10	34.6	5	27.9	6.7	0	0
20	1	10	33.7	5	28.5	5.2	1.5	1.34
20	2	10	34.5	5	29.5	5.0	1.7	1.90
20	3	10	34.5	5	29.9	4.6	2.1	2.32
20	4	10	33.0	5	28.9	4.1	2.6	2.68
20	5	10	33.9	5	30.5	3.4	3.3	3.60

* Calculated $1.34 \sqrt{\text{amount of enzyme}}$.
Room temperature throughout experiment 19 C.
Barometric pressure throughout experiment 752 mm.

TABLE 6
DEPENDENCE OF THE AMOUNT OF OXIDATION UPON THE TIME OF EXPOSURE TO THE OXIDASE

2% Hydrochinon (c.c.)	Enzyme Solution (c.c.)	Oxidized Shaken (min.)	Gas (c.c.)	Pipet Shaken (min.)	Gas (c.c.)	Difference in Amounts of Gas	
						c.c. Observed	c.c. Calculated*
20	1	5	33.9	5	27.5	6.4	6.44
20	1	10	33.9	5	27.8	6.1	6.06
20	1	15	34.1	5	27.7	5.4	5.42
20	1	20	33.8	5	29.3	4.5	4.52

* $6.57 - 0.128 \left[\frac{t}{5} \right]^2$

Room temperature throughout experiment 25.5 C.
Barometric pressure throughout experiment 754 mm.

DISCUSSION OF RESULTS

From the tabulated experiments the relations required by chemical dynamics for the simplest cases of catalytic reactions are found to be fulfilled by the action of the rhus enzyme on 2% hydrochinon: (1) approximate proportionality between the concentration of the enzyme and the velocity of the reaction (Duboscq colorimeter experiment); and (2) the validity of the rule $Ft=k$, indicating that the time required to achieve a given amount of oxidation is inversely proportional to the amount of ferment present.

It is interesting to note that the normal oxidation when combined with the accelerated oxidation is proportional to the square root of

the amount of enzyme when the temperature is 25.5 C. This result is the same as that which Slowtsoff¹⁸ obtained with potato laccase. There is a remarkable difference between this result and that obtained at 19 C. At 19 C. the result of the combined oxidations is more nearly proportional to the square of the amount of time. This difference seems unexplainable when compared with the rate of most chemical reactions, which is doubled or trebled for a rise of 10 degrees. This general rate may be applicable to the normal oxidation of hydrochinon separately, but the acceleration of this particular reaction due to the enzyme is greater at 19 than at 25 C., as shown in the beginning of this paper. At moderate temperatures the temperature coefficients of enzyme reactions are approximately the same as those of chemical reactions in general, but at temperatures in the neighborhood of 0 C.; the rate of change of the velocity constant with the temperature is often abnormally high, while at temperatures in excess of the optimum, that is, involving destruction of the enzyme, the velocity constant rapidly approaches zero. In the present instance the optimum temperature would appear to lie below 20 C.

THERAPEUTIC ACTION OF THE ENZYME SOLUTION

As the enzyme has the power to change the poison to a nontoxic substance while on the plant, it was thought that it might change the poison to a nontoxic substance when on the skin and thus be a remedy for rhus dermatitis. Experiments were conducted for me by Dr. Edward von Adelung to ascertain the value of the enzyme solution as: (1) Poisonous or not; (2) a preventative of rhus dermatitis; (3) remedy. The following results were attained: (1) The enzyme solution did not produce dermatitis tho rubbed briskly into the skin. (2) When mixed with rhus poison in alcoholic solution it did not destroy the poison. (The enzyme is active in 50% alcohol.) (3) It has no remedial value.

These results are as might be expected, for the enzyme is present in the expressed sap of the plant, and yet, when the expressed sap is rubbed on sensitive skin, dermatitis results. (Positive results for the presence of the enzyme in the sap may be obtained by mixing the sap with 6 times its volume of 95% alcohol. Filtering, dissolving the precipitate in distilled water, and testing with guaiac solution as on Page 6 of this thesis.) The enzyme is colloidal and will not pass through a semipermeable membrane, for example, parchment paper.

¹⁸ Ztschr. f. physiol. Chem., 1900, 31, p. 227.

The poison, on the other hand, is not colloidal as when in ethylacetate solution -the poison will pass through semipermeable membrane. Another evidence of the poison's ability to penetrate a semipermeable membrane is seen in its ability to penetrate the skin in the production of rhus dermatitis.¹² It is quite probable that the enzyme cannot penetrate the semipermeable skin and thus is unable to follow up and destroy the poison. Failure to show remedial properties in this enzyme solution, apart from its inability to penetrate normal skin, may be due to a matter of dilution. There are, however, oxidizers in the skin which seem to be incapable of the rapid destruction of the poison, for example, the leukocytes and nucleoprotein.¹⁹

SUMMARY

The browning of the sap of *Rhus diversiloba* is due to oxidation.

This oxidation results in the total loss of the irritating poisonous properties of the sap.

This oxidation is greatly accelerated naturally by an enzyme.

The oxidase accelerates the transfer of molecular oxygen to phenols, and aromatic amins.

The oxidase accelerates the oxidation of ortho- and para-isomeric phenols more greatly than metaphenols.

The oxidizing power of the enzyme is accelerated by dilute alkalis.

The oxidizing power of the enzyme is retarded by dilute acids.

The oxidizing power of the enzyme is destroyed when boiled for a short time in aqueous solution.

Rhus diversiloba oxidase is probably similar to that isolated from *Rhus vernicifera* by Yoshida and Bertrand. (Stevens and Warren²⁰ observed a thermolabile enzyme in *Rhus vernix*, probably an oxidase, which gave characteristic reactions with guaiac, alpha naphthol, and guaiacol.) It is a soluble ferment accelerating the oxidation of readily oxidizable bodies, particularly those of the benzene series, which possess at least two groups hydroxyl or amin when these groups occupy the para or ortho position.

The oxidase apparently has no therapeutic value either as a preventive or as a cure for rhus dermatitis.

¹⁹ There is another phenomenon that tends to explain the therapeutic failure of the enzyme solution namely, that the body may form an anti-enzyme specific in its inhibitive action against *rhus diversiloba* oxidase. Gessard (Compt. rend. Acad. d. sc., 1903, 136, p. 631; Compt. rend. Soc. biol., 1903, 55, pp. 227, 637) obtained a serum capable of retarding the action of the oxidase of *rhus vernicifera* by the subcutaneous injection into a rabbit of preparations containing this enzyme.

²⁰ Am. Jour. Pharm., 1907, 79, p. 499.

ANAPHYLATOXIN AND ANAPHYLAXIS*

I. TRYPANOSOME ANAPHYLATOXIN

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SYNOPSIS

FOREWORD

METHODS EMPLOYED

ANAPHYLATOXIN-PRODUCTION WITH:

SURRA TRYPANOSOME (T. EVANSI)

CADERAS TRYPANOSOME (T. EQUINUM)

DOURINE TRYPANOSOME (T. EQUIPERDUM)

TRYPANOSOMA LEWISI

SERIAL PRODUCTION

NAGANA TRYPANOSOME (T. BRUCEI)

GUINEA-PIG SERUM; SERIAL PRODUCTION; TOXIC WAVE.

RAT BLOOD AT 0 AND AT 37 C.; RAT SERUM AT 37 C.

EFFECT OF COLD AND HEAT. ATTEMPTS AT DETOXIFICATION.

ATTEMPTED DEMONSTRATION OF FERMENT ACTION.

COMPARATIVE EFFECT OF HIGH- AND LOW-SPEED CENTRIFUGATION.

MAXIMAL TOXICITY. APPARENT LOSS OF TOXICITY.

IN-VIVO PRODUCTION OF POISON IN INFECTED RATS.

SHOCK IN GUINEA-PIGS BY INJECTION OF WASHED TRYPANOSOMES.

INJECTION OF NORMAL SERA INTO NAGANA ANIMALS;

TOXICITY OF NORMAL RAT SERUM.

SUMMARY

FOREWORD

In the course of experimental work on immunity against the trypanosomes of surra and nagana it was found that severe toxic effects with marked hypothermia often followed the injection of autolyzed suspensions of the organisms. When such injections were repeated the chronic intoxication thus induced usually ended fatally, and while a certain minimal protection could be established in carefully treated animals, it was evident that the poisonous action observed was a bar to the production of a higher degree of immunity. As no advance seemed to be possible without a clear understanding of the nature of this intoxication, the trend of the investigation had to be changed into a new direction. At first the poisonous effects were identified with the 'trypanotoxin' of Laveran,¹ but the similarity to

* Received for publication December 5, 1916.

¹ Laveran et Pettit, Bull. Soc. path. exot., 1911, 4, pp. 42, 680.

that of the so-called anaphylatoxins was such that it led to a study of trypanosomes in their relation to the production of this poison.

Exactly similar conditions have been encountered in connection with the so-called bacterial 'endotoxins' from the time of Pfeiffer's² work on the cholera vibrio down to the more recent studies of Rosenow³ on pneumococci. Indéed, the bacterial anaphylatoxins have been identified with such endotoxins. The same problem reappears in recent studies on the toxicity of blood and of organ extracts of animals, and also of plant extracts.

The results obtained in this study of trypanosomes confirmed and extended previous work, but it was not sufficient to know that trypanosomes in some way give rise to anaphylatoxin. It was desirable to learn, if possible, the exact rôle played by the organism in the production of the poison, as well as the source and nature of the latter; in short, the mechanism of the reaction as a whole called for solution. As the work progressed it became necessary to apply similar questions to other of the so-called antigens, such as bacteria, agar, peptone, organ extracts, etc.; thence, the road led to the larger problem of anaphylaxis proper and even to the phenomenon of blood coagulation. It will be seen therefore that the work has extended well beyond its original scope, with the advantage that whenever an impasse was met in one direction another lead was available. Without doubt the results obtained have a direct bearing on the problems of immunity, which must be approached with a conception of the nature of the mechanism different from that which has hitherto prevailed.

The essential value of a theory lies in the stimulation it gives to the search for facts which serve for its support or rejection. But facts have often but a relative worth since their meaning and value change with the point of view. In biologic science especially, theories are but creations of the day, their place being taken by others of the morrow. The study of anaphylaxis has been fruitful of many theories, but it will be necessary to add a new one which, while unifying observations of utmost diversity, provides at the same time a basic conception regarding the interpretation of many pathologic and physiologic processes.

The demonstration that bacteria and diverse other organisms and cells, on contact with normal sera, give rise to anaphylatoxin quite naturally led several workers to test trypanosomes in this regard.

² Ztschr. f. Hyg. u. Infektionskr., 1894, 16, p. 268.

³ Jour. Infect. Dis., 1912, 11, pp. 94, 235, 286.

Friedberger and Szymanowski⁴ mentioned obtaining a poison from this source, but no details were given. Dold⁵ merely referred to this statement. The first actual publication dealing with the production of anaphylatoxin by trypanosomes was that of Marcora,⁶ who succeeded in obtaining one fatal anaphylactic shock in a guinea-pig by the injection of guinea-pig serum (4 c.c.) toxified by means of washed nagana trypanosomes. The mixture of trypanosomes and serum was kept at 37 C. for 2 hours, then in an icebox for from 12 to 18 hours, and afterward was centrifugated. Conforming with the prevailing theory, he believed that the poison was produced out of the trypanosomes. Mutermilch⁷ reported a brief series of tests with the same organism. By keeping a mixture of *T. brucei* and guinea-pig serum for from 2 to 3 hours at 38 C., then icing it for 20 hours or more, he obtained a toxic serum which killed in from 2 to 3 minutes in dose of 3 c.c.

METHODS EMPLOYED

At the outset and to a very large extent during the course of this investigation, the serum of white rats, either normal or infected, was employed, and to this fortuitous circumstance was due in large measure the success which attended this work. The great advantage of the serum of the rat over that of the guinea-pig, rabbit, etc., lies in the fact that it readily yields a very active anaphylatoxin which is fatal in dose of 0.5 and even 0.25 c.c. With such an active reagent at our disposal it became possible to attack the various problems as they presented themselves. Furthermore, the rat itself, as a reagent, was found to possess marked advantages over other animals, and this fact proved most useful, especially in the study of anaphylaxis.

To avoid unnecessary repetition it will be well to state, once for all, that all tests, unless otherwise indicated, were made under strictly sterile conditions. Obviously, the most important manipulation consisted in bleeding the animal. Here, as in many other things, practice was essential to success.

Rats were bled from the heart by means of a pipet such as is shown in Fig. 1. This was readily made from a test tube (16×160 mm.) by drawing out the end and bending the narrowed portion, the two operations being made at the same time. A glass rod, passing through the cotton, served to defibrinate the blood. The etherized rat was attached to a suitable board, the heart was then exposed, grasped with a special oval-tipped forceps, and the tip of the pipet, previously broken off and flamed, was then inserted. While mouth suction was applied to the tube, the capillary was passed back and forth into the auricle and venae cavae until the rat was exsanguinated. The tip of the pipet was then sealed in the flame. In special cases in which speed was necessary, the tip was closed with sealing wax or vaselin. The blood was then defibrinated for a few minutes, after which it was transferred with a bulb pipet or poured directly into a pooling flask, or centrifuge tube, previous flaming, of course,

⁴ Ztschr. f. Immunitätsf., 1911, 9, p. 379.

⁵ Das Bakterienanaphylatoxin, etc., 1912.

⁶ Ztschr. f. Immunitätsf., 1911, 12, p. 595.

⁷ Compt. rend. Soc. de biol., 1912, 73, p. 56. Ann. de l'Inst. Pasteur, 1913, 27, p. 37.

being resorted to. The actual bleeding time was from 1 to 1½ minutes, while the entire operation did not necessarily take over 5 minutes. The duration and mode of defibrination have an important bearing on the result; it should be as short as possible.

Guinea-pigs were also bled from the heart; the heart pipet in this case being made from a larger test tube (25×200 mm.). The manipulations otherwise were the same as given in the foregoing. When only a small amount of blood was desired, this was often obtained with a syringe, by direct heart-puncture, through the thoracic wall. In other cases, in which speed was essential, the blood was drawn from the exposed heart into a syringe.



Fig. 1. The Novy heart pipet used in bleeding rats, etc.

Rabbits were usually bled from the carotid by means of large pipets similar to that described. These were made by attaching a 10-mm. glass tube to the bottom of a large test tube (30 to 45 mm. in diameter by 200 mm.). The tube was then drawn and bent at a suitable angle, sealed, and provided with a cotton plug through which a glass defibrinating rod was passed. Just before use the tip of the sterile pipet was cut off and flamed, after which it was inserted into the exposed carotid. Direct heart-puncture by means of a syringe was resorted to when only a small amount of blood was desired. The Latapie holder was invaluable in work with rabbits and guinea-pigs.

The centrifugation of the blood, or of serum suspensions, was usually effected by means of an ordinary small electric centrifuge, carrying either 2

or 4 tubes, each having a capacity of 15 c.c. The speed of this machine on actual testing was found to be about 3000 revolutions per minute. The centrifuge tubes were plugged and sterilized before use.

In addition to this centrifuge, a larger one made by Burmeister and Wain, of Copenhagen, was found to be very useful. This machine has a maximal speed of 8000 revolutions per minute, each 100 revolutions being announced by a bell. It carries 6 tubes, each of 50-c.c. capacity, but smaller tubes can also be used. In order to keep the fluids sterile when the machine was operating at full speed, it was necessary to construct special centrifuge tubes. The revolving drum, it may be added, is 35 cm. in diameter and weighs 14 kilos. By means of this high-speed centrifuge it was possible to remove suspended matter to an extent otherwise impossible.

The incubation of sera was usually effected in a Roux water bath provided with a metallic regulator. As a rule the sera were placed in small Erlenmeyer flasks (30 to 50 c.c. capacity) so as to form a thin layer, thus being insured good contact between liquid and suspended matter.

The injection into the guinea-pig was always made in the external jugular vein, the animal being held on a Latapie board. The animal was then immediately released and the effects noted. The last faintest nasal twitch was taken as the death point. Individual variation in the susceptibility of the guinea-pig is to be borne in mind; a negative result does not prove the absence of the poison. It will be shown later on (Table 45), that not infrequently 2 guinea-pigs of equal weight, injected within a few seconds of each other, with the same material and dose, may behave totally differently; one may die of acute anaphylactic shock, while the other may show practically no effect. This, of course, holds true when approximately 1 lethal dose is given.

In the trypanosome work the animals were usually infected by intraperitoneal injection. Unless otherwise stated, they were bled shortly before death, at which time the number of trypanosomes approximated 200 per field of the No. 7 objective (eye-piece No. 3 being used). On centrifugation of such blood, the trypanosomes separated as a thick white layer on top of the erythrocytes. After the removal of the serum with a drawn-out bulb pipet, the organisms were taken up in a drawn pipet and transferred to a tube containing from 8 to 10 c.c. of 0.85% salt solution. Thorough agitation and recentrifugation, the operation being repeated 3 times, gave serum-free organisms.

The trypanosomes of surra, caderas, and dourine, and *T. lewisi* were employed in the earlier part of the work to show that anaphylatoxin-production is common to all of these, but the major part of the study concerned *T. brucei*. The relatively feeble toxicity of the sera treated with the first four organisms merely shows the poorness of the methods as first employed. With rat serum and short incubation they would without doubt give the same toxicity as serum treated with *T. brucei*; that is, a lethal dose of from 0.25 to 0.5 c.c.

SURRA TRYPANOSOME (*T. EVANSI*)

The study of anaphylatoxin began in connection with the work on immunity against surra. On the assumption that an anaphylatoxin was probably formed *in vivo* in the blood of infected rats, especially just before death, the sera obtained from such source were tested for the presence of this poison. The blood of the infected rats was defibrinated as quickly as possible, pooled, and centrifugated at 3000 revolutions for 5 minutes. The serum was then decanted and, usually, instead

of being injected at once, it was placed on ice for 4 hours; this in subservience to the prevailing opinion that the primary toxicity of normal sera is reduced to a minimum by being kept for some hours at a low temperature.

An unusual toxicity of the trypanosomal, as compared with normal, serum seemed to be suggestive of in-vivo production since the icing of such sera after the removal of the organisms did not result in a further increase. Neither was there any marked decrease in toxicity on icing, for the initial titer sometimes persisted for an indefinite period. Thus a pooled surra serum which was kept in the ice chest at about 5 C. for 171 days produced acute anaphylactic death in dose of 2 c.c. (Table 1, Guinea-pig 10).

The results of these tests with the serum of surra are given in Table 1. The serum used for Guinea-pigs 1 to 4 was from the pooled blood of 6 heavily infected rats (200 per field); that for No. 5 was from 3 rats, bled while dying; that injected into Guinea-pigs 6 to 8 came from 3 likewise heavily infected rats. The serum employed for Guinea-pig 9 came from a single heavily infected rat, and was injected after being kept in the room for 1 hour; Guinea-pig 10 also received the serum from a single rat, which showed an extremely heavy infection (300 per field); this serum it is to be noted was kept iced for 171 days.

TABLE 1
ANAPHYLATOXIN IN DEFIBRINATED BLOOD OF SURRA RAT, AND IN GUINEA-PIG SERUM (No. 11)

Guinea-Pig		Serum*		Result
No.	Weight	c.c.	Previous Treatment	
1	350	3.0	Iced 4 hr.	3½%; typical shock and autopsy
2	205	5.0†	" " "	Very slight
3	350	2.0	" " "	Severe
4	250	2.0	" " "	2%; typical shock and autopsy
5	300	5.0	" 3 "	Slight
6	265	4.0	" 4 "	Moderate
7	250	1.5	" " "	6%; typical shock and autopsy
8	212	0.75	" " "	Slight
9	250	2.0	Kept at 20 C., 1 hr.	17 15%; very severe shock
10	250	2.0	Iced 171 days	27 15%; very severe shock
11	260	4.5 (guinea-pig)	Kept at 37 C., 3 hr.; iced 24 hr.	Slight

* All injections were made intravenously except that of Guinea-pig 2.

† The injection of 2 was made intraperitoneally; 45 minutes later a second injection of 2 c.c. were given, without any effect.

KEY TO TABLES

In this and the following tables, the expression "typical shock" implies the full syndrome noted in anaphylaxis, namely: peripheral irritation, dyspnea, spasms, convulsions, urination. Likewise, "typical autopsy" means the findings of specific anaphylactic death, namely: permanent maximal distension of lungs, heart beating, low blood pressure, blood fluid, no clot.

The nonfatal results are conveniently indicated in the following manner:

Very slight.—The only effect is a slight change in respiration.

Slight.—Respiration irregular, no dyspnea, few jerky spasms.

Moderate.—Respiration irregular, some dyspnea, few jerky spasms.

Fair.—Some dyspnea and marked spasms.

Good.—Some dyspnea and marked spasms with few slight convulsions.

Severe.—Spasms, convulsions, dyspnea, thrown momentarily.

Very severe.—Same but remains thrown for some time; no marked apnea.

Near-kill.—Same with prolonged suppression of respiration and apparently dead. The time of death is indicated in the tables.

These preliminary tests seemed to confirm the assumption that the poison was present during life in the blood of the infected rats, but subsequent work showed that most of the toxicity observed in this and similar series of tests was really due to in-vitro changes. Hence the results given in this and the following tables are to be interpreted in that sense. It is to be noted that the surra serum in one test (No. 7) was fatal in as small an amount as 1.5 c.c., which at the time was the smallest toxic dose for any anaphylatoxin. Experience and a better understanding of the reaction have made it possible to produce with *T. brucei* within 15 minutes a serum having a lethal dose of from 0.25 to 0.5 c.c. On the other hand, it will be seen that at times relatively large doses, up to 5 c.c. (Guinea-pigs 2, 5, and 6), may be given with little or no more effect than follows the injection of normal rat serum.

In view of the results obtained with the caderas trypanosomes it would seem that the surra serum is less toxic. This conclusion, however, is not justified, for subsequent work with *T. brucei* has shown that the toxicity depends on the temperature and the duration of contact of the trypanosomes with the defibrinated blood or serum, and even on the mode of defibrination. In the surra tests the infected rats were bled late at night, when the room temperature was relatively low as compared with that of the afternoon, when the caderas blood was obtained. As indicated in the foregoing more toxic sera can be obtained by keeping the defibrinated trypanosomal blood for a few minutes at 37 C.

Only one attempt was made to render normal guinea-pig serum toxic by bringing it into contact with the washed parasites. For this purpose the trypanosomes from 6 heavily infected rats, after being washed 4 times with salt solution, by centrifugation, were added to 4.5 c.c. of normal serum in a small flask. The mixture was placed at 37 C. for 3 hours, then iced for 24 hours, after which it was centrifugated and injected into Guinea-pig 11 (Table 1). The result was practically nil.

The outcome probably would have been different had the injection been made immediately after the incubation as in the case of like tests with the caderas trypanosome (Table 2) and *T. lewisi* (Table 5).

CADERAS TRYPANOSOME (*T. EQUINUM*)

This organism was found to produce anaphylatoxin in rat serum about as readily as did that of nagana. The mere keeping of the defibrinated blood at room temperature for about an hour was sufficient to give rise to considerable toxin, as will be seen from the following experiment: Five white rats inoculated with *T. equinum* were bled 8 days later, at which time they showed 200 or more trypanosomes per field. The blood was defibrinated, pooled, and centrifugated at 3000 revolutions for 5 minutes. The serum was pipetted off and placed on ice for 16 hours, after which it was tested with the results shown in Table 2. The interval from the start of bleeding to the icing of the serum was 1 hour.

TABLE 2
ANAPHYLATOXIN IN DEFIBRINATED BLOOD OF CADERAS RAT (1 TO 4), AND IN GUINEA-PIG
SERUM (5 AND 6)

Guinea-Pig		Serum* (c.c.)	Result
No.	Weight		
1	250	2.0	2'; typical shock and autopsy
2	230	1.0	3'; " " " "
3	250	0.5	7/30'; " " " "
4	240	0.25	Severe
5	250	4.5 (guinea-pig)	45'; at first severe convulsions; autopsy typical
6	240	4.8 (guinea-pig)	2'; typical shock and autopsy

* Intravenously injected.

It will be seen from Table 2 that even 0.5 c.c. of the serum produced an acutely fatal shock. The icing of the serum in this and other experiments is of no particular value, the essential factor in the poison-production being the temperature and time during which the trypanosomes act on the serum. These conditions were particularly established with *T. brucei* and probably hold true for the other organisms of this type.

The deposit of trypanosomes obtained after centrifugation in this experiment was washed with 8 c.c. of 0.85% salt solution and then iced over night. The next day the operation of washing with salt solution was repeated 3 times, after which the trypanosomes were added to a small flask containing 5 c.c. of fresh normal guinea-pig serum. The latter was obtained by centrifugation of blood drawn by heart-puncture.

The mixture of serum and trypanosomes was placed at 37 C. for 14 hours and then centrifugated. This serum was tested on a guinea-pig (No. 5). The trypanosomes were now added to a second portion of 5 c.c. of normal guinea-pig serum and incubated at 37 C. for only 4 hours. After centrifugation, the serum was tested with the result shown in Table 2, No. 6.

The minimal lethal dose of the sera used in Tests 5 and 6 was not determined for obvious reasons. Consequently it may be questioned as to how much of the effect observed was due to the primary toxicity of serum and how much to the anaphylatoxin formed by the trypanosomes. While in general it is true that normal guinea-pig serum in dose of 4 to 5 c.c. gives little or no effect, it is nevertheless a fact, which our subsequent experience established, that, at times, 4 c.c. and even 3 c.c. of normal serum may kill. It is wrong to assume that a normal serum such as that of the guinea-pig or rat is innocuous in the doses given. This and many other like experiments were made before it was realized that a normal serum was subject to variation, as is also the recipient. A sufficient amount of pooled serum to permit duplicate control tests as well as duplicate tests with the treated serum is really needed to obtain results which will be reasonably certain.

It may be deduced from these experiments that the caderas trypanosome produces anaphylatoxin in rat serum and probably also in that of the guinea-pig. It is also possible that a prolonged incubation of the serum trypanosome mixture may yield a less toxic serum.

DOURINE TRYPANOSOME (T. EQUIPERDUM)

Only a limited number of tests were made with the sera of animals infected with this organism, sufficient to fix the general fact that it like other trypanosomes gives rise to anaphylatoxin. No attempt was made to induce toxicity in normal serum by the addition of washed dourine parasites. These tests like the preceding were made early in the course of the investigation before the importance of the incubation at 37 C. was ascertained, and while we were still looking for evidence of the presence of poison during life. Consequently, the results given in Table 3 represent merely the toxicity developed in the serum during the process of defibrination and centrifugation, which were carried on at room temperature. The icing of the clear serum, as indicated before, was probably without any effect as regards increasing the toxicity.

Five rats, showing about 200 trypanosomes per field, furnished the material for Guinea-pigs 1 to 4. After defibrination the pooled blood was centrifugated and the clear serum iced for 4½ hours before injection. For Guinea-pig 5, the blood of only 1 rat was used (100 per field); the serum was kept at room temperature for 2 hours and then injected. For No. 6, a guinea-pig inoculated 30 days before and showing only a moderate number of trypanosomes (20 per field), was used. The result in this case was a moderate shock with a drop in temperature to 34 C.

TABLE 3
ANAPHYLATOXIN IN DEFIBRINATED BLOOD OF DOURINE RAT AND GUINEA-PIG

Guinea-Pig		Serum*		Result
No.	Weight	c.c.	Previous Treatment	
1	240	1.0	iced 4 hr.	Very slight
2	205	2.0	" " "	Moderate
3	210	2.0	" " "	"
4	230	3.0	" " "	2'; typical shock and autopsy
5	230	1.7	Kept at 20 C., 2 hr.	50'; " " " "
6	250	4.8 (g.-pig)	iced 4 hr.	Moderate

* Intravenously injected.

The tests recorded in Table 2 show that serum obtained under the conditions of the experiment possesses but a moderate degree of toxicity. The difference between these results and those gotten with caderas serum (Table 2) is striking, tho the conditions were much alike. It does not, however, mean that there is a real distinction between the two organisms concerned, but rather serves to emphasize the importance of such factors as temperature and duration of contact of the trypanosomes with the serum.

LEWIS TRYPANOSOME (T. LEWISI)

After preliminary experiments had shown that the pathogenic trypanosomes (surra, caderas, dourine, nagana) produced anaphylatoxin in rat serum, and that some of these organisms when washed were capable of toxifying normal guinea-pig serum, it was desirable to ascertain whether the relatively harmless *T. lewisi* was able to give rise to the same poison. The first tests with this material were decidedly negative and it seemed as if the poison-production was limited to the pathogenic forms. Subsequently, however, this was found to be incorrect. Given the right conditions, this organism is able to produce poison just as well as the pathogenic *T. brucei*, a fact which will be

seen on comparison of Tables 5 and 6. This certainly holds true for guinea-pig serum and seemingly should be equally true for that of the rat.

The first failures to produce the poison in rat blood (Table 4) are subject to several interpretations, such as that they were due to the small number of organisms present and perhaps to the fact that these parasites do not disintegrate as readily as other forms. The absence of incubation, at 37 C., is clearly a decisive factor. Noteworthy is the fact that relatively large doses of rat serum (6 c.c.) obtained under these conditions may be innocuous, but it must not be inferred that normal rat serum is always as harmless when given in such doses.

The serum used for Guinea-pig 1 (Table 4) was from the pooled blood of 4 rats (30 per field); that employed for No. 2 was from a pool given by 6 rats (75 per field); that for No. 3 was from 3 rats (100 per field); while that injected into Nos. 4 and 5 came from 3 rats (150 per field). In each case the pooled blood was centrifugated at 3000 revolutions for 5 minutes, after which the serum was iced for the time indicated.

TABLE 4
ANAPHYLATOXIN IN DEFIBRINATED BLOOD OF RAT INFECTED WITH *T. LEWISI*

Guinea-Pig		Serum*		Result
No.	Weight	c.c.	Previous Treatment	
1	235	6.0	Iced $\frac{1}{2}$ hr.	Nil
2	250	6.0	" " "	"
3	250	4.8	" " "	"
4	260	5.2	" 12 "	Severe
5	205	4.6	" " "	15'; typical shock and autopsy

* Intravenously injected.

That the toxifying power of *T. lewisi* is not inferior to that of *T. brucei* is clearly demonstrated in the following series of tests (Table 5) with normal guinea-pig serum. In this series the same mass of washed trypanosomes was used successively to induce anaphylatoxin-production in 14 different lots of such serum. The trypanosomes were obtained from the blood of 3 rats, the serum of which was used for Tests 4 and 5, recorded in the preceding table. After removal of the serum the organisms were washed 3 times with 0.85% salt solution, and centrifugated each time at 3000 revolutions for about 2 minutes.

The washed trypanosomes were then added to 5 c.c. of fresh normal guinea-pig serum, in a small flask, and placed at 37 C. for 4 hours. The mixture was then centrifugated, and the serum removed and tested

(No. 1). The organisms were then added to another portion of 4 or 5 c.c. of fresh serum, mixed, incubated, and centrifugated as before. This second serum was then injected (after being iced over night) into Guinea-pig 2. This operation was repeated until 14 consecutive tests had been made. The experiment was then discontinued. Seemingly, the operation could have been repeated, with like results, an indefinite number of times.

TABLE 5
SERIAL PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIG SERUM BY WASHED *T. LEWISI*

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Previous Treatment with <i>T. lewisi</i>	
1	225	4.5	4 hr. at 37 C.	1'30"; typical shock and autopsy
2	250	5.0	" " " " "	Very slight
3	245	4.3	and 12 hr. on ice	
4	225	5.0	4 hr. at 37 C.	2'; typical shock and autopsy
5	180	3.8	" " " " "	3'; " " " "
6	200	3.8	" " " " "	3'; " " " "
7	205	4.5	2 " " " "	2'; " " " "
8	180	3.0	2 " " " "	2'; " " " "
9	260	3.8	1 " " " "	2'; " " " , no autopsy
10	205	4.0	1 " " " "	2'; " " " and autopsy
11	250	4.0	1/2 " " " "	1'30"; " " " "
12	260	4.0	" " " " "	5'; " " " "
13	250	4.0	" " " " "	5'; " " " "
14	230	4.0	" " " " "	2'; " " " , no autopsy

* Intravenously injected.

It is noteworthy that the same mass of Lewis trypanosomes can be used to produce anaphylatoxin in guinea-pig serum for a considerable number of times. The results correspond exactly to those obtained with *T. brucei* (Tables 6 and 7). Similar serial toxifications with *B. pyocyaneus*, *B. typhosus*, etc., were obtained by Dold and Aoki,⁸ but in their case the reaction seemed to come to an end with about the 10th test. In the light of subsequent facts it is indeed very doubtful whether there is any real end to the inducing power of bacteria or of trypanosomes, other than the exhaustion of the energy necessary for the induction.

Another important fact shown in Table 5 is that contact of the trypanosomes with the serum at 37 C. for one-half hour is apparently just as effective as a contact of 4 hours. Without doubt a shorter time would have sufficed, for in a similar series of tests with *T. brucei* (Table 6) a period of 15 minutes was enough. It is clear therefore that at a suitable temperature the reaction is developed at considerable speed. In view of the action of distilled water on normal rat serum

⁸ Ztschr. f. Immunitätsf., 1913, 18, p. 207. Levy and Dold, *ibid.*, 1913, 19, p. 306.

(Part IX), it is likely that the optimal temperature will be found at or below 45 C. A series of tests with trypanosomes and rat serum at, or near, this temperature should give a maximal speed with the lethal dose well within 1 c.c.

NAGANA TRYPANOSOME (*T. BRUCEI*)

T. brucei was selected for the major part of this study, largely because of the intense infection which it produced in white rats within 2 or 3 days after inoculation. Consequently, a large amount of trypanosomes could be readily obtained from the blood after defibrination and centrifugation. At first the method of testing for anaphylatoxin was the same as that used with the surra trypanosomes and the other organisms, but later the icing of the trypanosome mixtures was abandoned in favor of continuous incubation and periodic testing.

Guinea-Pig Serum.—For a relatively small part of the work, normal guinea-pig serum was employed. Altho this is the most common reagent for anaphylatoxin-production, it is by no means the best, and hence in the course of time it was replaced almost entirely by the more responsive rat serum.

A few early tests indicated that normal guinea-pig serum could be toxified by *T. brucei* derived from infected rats. Thus, the trypanosomes obtained by centrifugation of the blood of 6 rats, after being kept for 7 days, iced and in contact with the cell deposit, were added to 5 c.c. of normal guinea-pig serum, incubated for 4 hours at 37 C. and iced over night. After centrifugation this treated serum, injected into a 205-gm. guinea-pig, caused typical shock with death in 2 minutes. The same trypanosomes were then treated with fresh guinea-pig serum in exactly the same way, and this serum in dose of 5 c.c. was also fatal to a 260-gm. guinea-pig in 3 minutes, the symptoms and findings being typical. A control test made at this time with 5 c.c. of normal guinea-pig serum, incubated and iced just as in the test proper, produced no effect. After a lapse of 7 days, the same trypanosomes were again treated with guinea-pig serum, as before, but this time the serum in dose of 5 c.c. produced only a severe shock. In these tests the same organisms apparently toxified the normal serum 3 times in succession. It might be objected that the effects observed, in spite of the one control test, might be due to a primary toxicity of the serum, since the dose employed was rather large.

To exclude such error it was necessary to inject smaller doses, and this condition was gradually met as seen from the following tables.

For the experiments recorded in Table 6 the blood of 4 rats, inoculated 3 days before and rich in parasites (200 per field), was used. The organisms were drawn off, after centrifugation, and washed with 8 c.c. of salt solution, and this washing was repeated 3 times. After final centrifugation they were added to 5 c.c. of normal guinea-pig serum, in a small flask, and incubated in the Roux water bath at 37 C. for the time indicated in the table. The clear serum obtained by centrifugation was then tested, while the trypanosomes were treated again with fresh serum. The experiment extended over 7 days and was then discontinued because it was deemed best to start a new series with a larger number of daily treatments. When not in actual use, the organisms were kept iced. There was no evidence of bacterial contamination. It will be seen from Table 6 that the toxifying power of the trypanosomes was maintained through 14 consecutive treatments with guinea-pig serum, the same as in the case of *T. lewisi* (Table 5). Even 15 minutes at 37 C. is sufficient to toxify the serum.

TABLE 6
I. SERIAL PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIG SERUM BY WASHED *T. BRUCEI*

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Previous Treatment with <i>T. brucei</i>	
1	250	4.5	2 hr. at 37 C.	Severe shock
2	260	5.0	" " " "	3'; typical shock and autopsy
3	210	5.0	" " " "	1'30"; " " " "
4	250	3.8	" " " "	2'; " " " "
5	200	4.0	" " " "	3'; " " " "
6	250	3.8	1 " " " "	2'; " " " "
7	200	3.8	" " " "	4'; " " , no autopsy
8	260	4.5	1½ " " " "	2'; " " and autopsy
9	260	3.0	¼ " " " "	Fair shock
10	300	4.0	¼ " " " "	2'; typical shock
11	200	4.0	" " " "	2'; " " and autopsy
12	230	4.5	¼ " " " "	3'; " " "
13	230	4.5	" " " "	3'; " " "
14	205	3.3	50' in room	4'; " " and autopsy

* Intravenously injected.

The trypanosomes for the next series were obtained from the blood of 4 rats inoculated 2 days before. The washing with salt solution and the further treatment were the same as described heretofore. The results are found in Table 7. In order to save animals no injections were made with Sera 10 to 13 and 15 to 19, but the results obtained with 14 and 20 show that the toxifying power of the organisms was kept up. The number of treatments, it will be seen, is double that reached by Dold and Aoki,⁸ and it is probable that a great many more would have given like results. Altho this experiment extended over 8 days, the material showed no bacterial contamination.

TABLE 7

II. SERIAL PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIG SERUM BY WASHED T. BRUCEI

Guinea-Pig		Serum		Result
No.	Weight	e.e.*	Previous Treatment with T. brucei	
1	200	3.5	½ hr. at 37 C.	Severe shock
2	210	3.5	" " " " "	3'
3	215	3.0	" " " " "	2'
4	230	"	" " " " "	4'
5	250	"	" " " " "	Slight shock
6	250	"	" " " " "	Very slight
7	240	"	4 " " " "	4'
8	220	"	12 hr. at room temperature	3'
9	220	"	2 hr. at 37 C.	3'
14	230	"	" " " " "	No tests made of Treated-sera 10 to 13 inclusive
20	170	"	" " " " "	No tests made of Treated-sera 15 to 19 inclusive
				3'30"

* Intravenously injected.

Occasional failure to kill, as in Guinea-pigs 1, 5, and 6, must be expected, since the conditions are never exactly alike. Guinea-pigs do show individual resistance to anaphylatoxin, and sera from animals of the same species have been found to vary. The question of an initial toxic wave is also to be considered. When the same pooled serum is used for all the tests of a series, as in Table 8, the cause of failure is reduced either to individual variation on the part of the guinea-pig or to a change in the toxic state. If the amount employed represents more than 1 lethal dose, obviously the failures will be less in number (see Table 17).

TABLE 8

TOXICITY OF GUINEA-PIG SERUM TREATED WITH WASHED T. BRUCEI AND TESTED AT INTERVALS

Guinea-Pig		Incubation of Treated Serum at 37 C. (hr.)	Result of Intravenous Injection of 3 e.c.
No.	Weight		
1	172	1½	Practically nil
2	175	1½	Slight
3	205	¾	3'10"; typical shock
4	210	1	Slight
5	195	1¼	Very severe shock
6	187	1½	Very slight
7	178	1¾	Fair

For the experiment recorded in Table 8, the blood of 3 nagana rats, after defibrination, was pooled and centrifugated at 3000 revolutions for 10 minutes; the trypanosomes were then removed and washed once with salt solution. They were then added to 23 c.c. of fresh normal guinea-pig serum, and the mixture was placed in ice for 1 hour. It

was then put at 37 C., and at every quarter hour a portion was removed, centrifugated, and injected. It will be noted that a fatal dose of poison apparently was not formed until after incubation for 45 minutes, whereas in the tests recorded in Tables 6 and 7 this occurred at times in 30 and even in 15 minutes. This difference may find an explanation in the relative amounts of organisms and sera used in these experiments.

An apparent oscillation in the toxicity is indicated in the results as tabulated, and this feature is brought out more clearly in Chart 1. This and similar charts must be interpreted as expressing merely the results of the experiment, for if the injections had been made in duplicate at each period some variation undoubtedly would have been found. In other words, the curve indicates not so much a variation in the amount of the poison as a variation in the resistance of the animals.

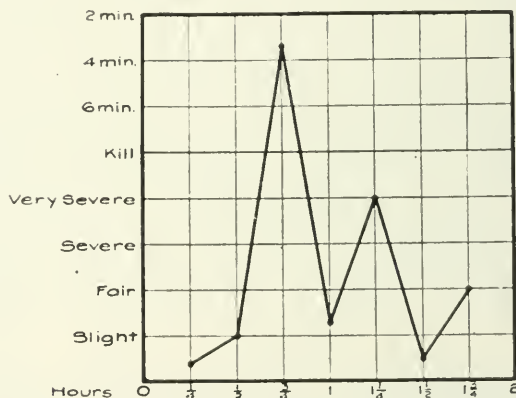


Chart 1. Apparent variation in toxicity of guinea-pig serum tested with *T. brucei* (Table 8); for meaning of terms, see Table 1.

Rat Blood and Serum.—In the early tests with infected rat blood, this was defibrinated at room temperature, pooled and centrifugated, the serum removed and iced for 4 hours, and then its toxicity tested. The great influence of time and temperature was not recognized until later. The first object here, as with the other trypanosomes, was to ascertain whether this organism gives rise to anaphylatoxin within the body of the rat. As seen from Table 9, which includes such early tests, it was readily shown that anaphylatoxin is produced in nagana rat blood, but whether all or a part or none of this exists *in vivo* remained to be determined. Further work showed that probably all of the results given in this table were due to the poison which is formed *in vitro*. A more certain and delicate method of detecting the presence of poison within the body was elaborated in time and will be taken up later on.

Table 9 includes the results obtained with 7 pools of nagana blood, the serum from each of which was treated as indicated. Pool 6 was obtained from 5 rats which were bled within an hour of death and consequently were extremely rich in organisms. On the other hand, Pool 4 was derived from the blood of 2 rats only moderately rich in parasites (50 per field). Pools 1 and 2 were also each obtained from 2 rats, but these were very rich in parasites (200 per field); Pools 3 and 7 came each from 6 rats equally rich in trypanosomes. Pool 5 was derived from 3 rats which were bled on the day following inoculation; at that time they showed only about 25 organisms per field.

TABLE 9
ANAPHYLATOXIN IN DEFIBRINATED BLOOD OF NAGANA RATS; SERUM KEPT AT 0 C. FOR 4 HOURS

Guinea-Pig		Serum		Result
No.	Weight	Pool	c.c.*	
1	215	1	2.0	4'; typical shock and autopsy
2	225	"	4.0	1'30"; " " " "
3	240	2	1.0	1'30"; " " " "
4	250	"	2.0	2'30"; " " " "
5	305	3	0.5	12'; " " " "
6	230	"	1.0	3'; " " " "
7	220	"	2.0	4'; " " " "
8	250	4	2.0	Nil
9	250	"	2.0	"
10	240	5	4.0	6'; typical shock and autopsy
11	240	6	0.25	Nil
12	250	"	0.50	Slight
13	240	"	1.0	"
14	250	"	4.0	4'; typical shock and autopsy
15	210	7	0.5	Slight
16	230	"	1.0	2'; typical shock and autopsy

* Intravenously injected.

Reference to Table 9 will show that the fatal dose ranged from 0.5 to 4 c.c. In view of the fact, which was recognized in time, that rat serum is very readily toxifiable, it is to be expected that in dose of 4 c.c. it would prove fatal. The extent to which anaphylatoxin is produced depends chiefly on the temperature and duration of operations preceding the transfer to ice. In all work with serum it must be remembered that the primary toxicity which is always present is merely increased by an external agent, in this case the trypanosome. Hence large doses of any treated serum are likely to give fatal results, since the toxicity of such serum represents the sum of primary and newly made anaphylatoxin. While it is possible to inject as much as 6 c.c. of rat serum without any effect, it is equally true that a slight variation in the method of defibrination may yield a serum which will kill in half that dose (see p. 506).

The influence of temperature on the formation of anaphylatoxin is clearly shown in Table 10. For these experiments the rats were bled on the second or third day of the infection, when the blood showed about 200 organisms per field. Each test was made with the blood of a single rat, except those the numbers of which are qualified as 8 and 8a, which indicates that they have a common blood source.

In the first two tests, the blood as soon as drawn was transferred to a centrifuge tube and immediately swung; the resulting serum was injected at once. It will be seen that such serum is practically without effect in the dose employed; that is, at this point no appreciable amount of poison is present.

It is possible that a much larger amount of such serum might have produced a toxic result, but it is self-evident that in the live, apparently well, tho heavily infected, animal the entire blood cannot carry much poison, otherwise a sudden and fatal intoxication would necessarily result. This may indeed be what happens in the terminal stages of the disease, especially in the guinea-pig, which is very susceptible to anaphylatoxin. The rat, however, which is insusceptible to enormous doses of anaphylatoxin, as will be later shown, may very easily carry such poison in its blood. As a matter of fact, transfusion experiments (Table 18) make it highly probable that some anaphylatoxin is present in the blood of infected rats.

In Test 3, the blood was spontaneously clotted in a centrifuge tube and kept at 37 C. for 1 hour. This serum produced a fatal shock with typical findings. For Guinea-pig 4 the blood was defibrinated for 1 minute and at once centrifugated; this serum again had but slight effect. The same was true for Tests 6 and 7, in which the defibrinated blood was kept at 37 C. for 2½ minutes. On the other hand, the incubation of the defibrinated blood for 5 minutes or more yielded a serum which was toxic in dose of 1 c.c.

A better result than the one just mentioned was shown in Test 5, which was made 2 years after the others in this table, with the express purpose of demonstrating the rapid speed of production. In this case the blood of a nagana rat (150 per field) was defibrinated in a water bath at 38 C. for 2 minutes, after which it was centrifugated for 4 minutes and the serum injected. The total time from the start of the bleeding to the end of the injection was 10 minutes. Several tests made at this time with blood of nagana rats, which was defibrinated for 1 minute in a bath at 38 C. and then centrifugated either at once or after

further incubation for 1 or 2 minutes, gave little or no result; the blood incubated for 3 minutes, however, gave a serum which killed in dose of 2 c.c. in 11 minutes 30 seconds. It would appear from this as if the duration of the defibrination at 38 C. — that is, the agitation — was a factor to be considered in the production of anaphylatoxin.

The foregoing experiments demonstrate that the anaphylatoxin-production in trypanosomal blood occurs in vitro and, under favorable conditions of temperature, is but a matter of a few minutes. Normal rat blood, defibrinated or whole, and incubated at 37 C. for $\frac{1}{4}$, 1, 3, 6, and 12 hours, produced no effect when injected in dose of 1 c.c. When incubated for a much longer time, normal rat serum may, however, become toxic in this dose.

TABLE 10
ANAPHYLATOXIN IN WHOLE DEFIBRINATED BLOOD OF NAGANA RATS

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Previous Incu- bation at 37 C.	
1	210	1.0	0	Nil
2	230	"	0	Very slight
3	220	"	1 hr.	3'; typical shock and autopsy
4	205	1.0	1'	Slight
5	205	" †	2'	5'20"; typical shock
6	203	"	2'30"	Very slight
7	205	"	"	Fair shock
8	210	"	5'	3'
8a	200	0.5	"	Slight
9	250	1.0	15'	5'
10	190	0.5	1 hr.	4'
10a	205	0.25	"	Slight
11	195	1.0	"	3'50"
11a	200	0.5	"	Slight
12	200	"	2 hr.	4'20"
12a	190	0.25	"	Severe
13	220	1.0	4 hr.	3'30"
13a	180	0.5	2 hr.	Slight
14	200	1.0	6 hr.	3'30"
14a	205	0.5	"	Good shock
15	200	1.0	12 hr.	4'
15a	175	0.5	"	2 hr.
16	200	1.0	"	5'
16a	208	0.5	"	Severe

* Intravenously injected.

† Defibrinated at 38 C.

Since it had been shown that practically all of the anaphylatoxin developed in nagana rat blood was formed after the latter was drawn, it was next in order to demonstrate that washed *T. brucei* in contact with normal rat serum likewise gives rise to the poison. An early series of tests made with this object in view is recorded in Table 11. For a given test the blood of a highly infected rat was at once chilled in ice water, then defibrinated and centrifugated. After removal of the

serum the trypanosome layer was taken up with salt solution and again centrifugated. The deposit was then washed thrice with salt solution and finally it was taken up with 2 c.c. of normal rat serum and this mixture was then incubated for the time indicated. The serum after final centrifugation was injected in the dose indicated.

It will be seen that such mixtures may become toxic within 1 hour and that even 0.5 c.c. of such treated serum may be fatal. Tests 4, 5, and 8 were made with separate mixtures; Nos. 1, 2, and 3 were made with one mixture, while for Nos. 6 and 7 another was used. For Tests 6 and 7 the mixture, after incubation, was iced for 12 hours, and this fact may in part explain the failure to kill. For Test 5 the trypanosomes were not fresh, but had been iced in contact with the erythrocyte deposit for 6 days.

Even more marked results than those here given were obtained when, after centrifugation of the infected blood, the deposit of trypanosomes was taken up with its supernatant serum. Such tests were frequently resorted to, as will be seen in the following pages. Especial reference, however, may be made to Table 17.

TABLE 11
ANAPHYLATOXIN PRODUCED IN NORMAL RAT SERUM BY WASHED T. BRUCEI

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Previous Treatment with T. brucei at 37 C.	
1	205	1.0	1 hr.	4'
2	215	0.5	"	3'30"
3	210	0.25	"	Severe shock
4	190	1.0	"	3'
5	220	"	"	Moderate shock
6	205	"	4 hr. and 12 hr. at 0 C.	Severe "
7	190	"	" " " " "	" "
8	200	"	12 hr.	3'30"

* Intravenously injected.

Effect of Cold.—It has been shown heretofore that a surra rat serum, kept in the ice box for 171 days, was toxic in dose of 2 c.c. This result led to a series of tests with nagana serum under like conditions. The results are given in Table 12, from which it will be seen that the toxicity was unimpaired for 24 days. The final test made with the last portion of the serum on the 35th day gave a severe shock, but did not kill a guinea-pig of 300 gm. weight.

For this experiment the blood of 6 nagana rats was pooled, centrifugated for 5 minutes, and the serum transferred to a plugged, but not otherwise sealed, test tube and kept iced.

Effect of Heat.—The question of the production of anaphylatoxin by heated trypanosomes and by inactivated sera will be considered later. At this point it is desired to indicate the effect of heat on the anaphylatoxin formed by *T. brucei* in rat serum. In common with the anaphylatoxin of other origin, this poison is apparently unaffected by exposure to 60 C. Thus, a nagana serum the minimal lethal dose of which was 1 c.c. was placed in a Roux water bath at 60 C. for 1 hour. One cubic centimeter of this heated serum produced typical shock and death in 2½ minutes in a guinea-pig weighing 220 gm. When it was added to an equal volume of normal rat serum and incubated for 1 hour at 37 C., there was no increase in toxicity; the inducing power, whether due to ferment or to residual trypanosomal matter, had been lost or impaired.

TABLE 12
PERSISTENCE OF ANAPHYLATOXIN IN ICED SERUM FROM NAGANA RATS

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Iced	
1	230	1.0	4 hr.	2'
2	210	0.5	" "	Slight
3	240	1.0	16 "	2'30"
4	230	0.5	20 "	Moderate
5	225	1.0	40 "	3'
6	250	"	3 days	3'30"
7	250	"	4 "	3'
8	270	"	7 "	3'
9	230	"	14 "	3'
10	250	"	24 "	2'
11	300	"	35 "	Very severe

* Intravenously injected.

Since a serum on prolonged heating at 60 C. or at a higher temperature is soon gelatinized, it is not so easy to determine the action of such. If the serum can be rendered noncoagulable, without interfering with the test proper, then the action at these higher temperatures can be ascertained. This can be done by diluting the serum with several volumes of water, a feasible procedure in the case of rat anaphylatoxin having a lethal dose of 0.5 c.c. or less. A better method is one which has been in use in this laboratory for many years; this is based on the fact that a serum when dialyzed in a collodium sac at 50 C. for 5 minutes or more is noncoagulable even upon boiling. The increase in volume depends on the thickness of the sac and on the duration of the dialysis. Thus, in one test 1 c.c. of toxic serum representing a fatal dose was dialyzed, first at 50 C. for 15 minutes, then at 70 C., for

30 minutes. The total bulk, amounting to 2.25 c.c., injected into a guinea-pig (220 gm.) produced no effect.

This experiment goes to show that at 70 C., in half an hour, at least 1 lethal dose is destroyed. In Part IV, under the heading "Heating of the Mixture to 60 C. before Incubation," will be found tests on the agar anaphylatoxin from rat serum which show that at 70 C. for half an hour the poison is but partially destroyed. As to the identity of the anaphylatoxins prepared with trypanosomes and with agar there can be no doubt.

Attempts at Detoxification.—In line with previous observations of others, it will be shown later that freshly drawn blood becomes intensely toxic in the precoagulation stage and that after clotting occurs there is a marked decrease in toxicity. Normal sera, however, remain poisonous tho the toxicity of a given specimen may apparently vary, especially when judged by single animal tests. Since the sudden toxic wave inaugurated by the coagulation changes rapidly recedes, it was thought that the addition of normal blood, immediately after being drawn and before coagulation started, to anaphylatoxin might effect a reversal or destruction of the latter.

Several attempts were made in this direction by adding varying amounts of fresh guinea-pig blood (1 to 6 c.c.), obtained by heart-puncture, to a single lethal dose (1 c.c.) of toxic rat serum. After clotting, the mixture was allowed to stand for 1 hour, either at room temperature or at 37 C.; it was then centrifugated and the resulting serum injected into guinea-pigs. In each test, typical shock and death occurred in 3 minutes. Similar tests made by adding varying amounts of normal rat serum (1 to 3 c.c.) to the lethal dose of toxic serum (1 c.c.) likewise resulted fatally. It would appear therefore that neutralization of the poison does not occur under these conditions. A repetition of these tests, with a more toxic serum and without the incubation, may be desirable in view of the fact that the toxicity of organ extracts is influenced by normal sera. The toxicity of the extracts, however, is not to be considered as due to preformed poison, but to the anaphylatoxin to which they give rise in vivo on injection.

Several attempts were made to detoxify rat serum by adding a small amount of guinea-pig bile. In 2 of 5 trials, the mixtures apparently became nontoxic. However, unless such tests are made in duplicate, side by side, to exclude individual variability of the guinea-pigs, they can have but little value (see Table 45). Thus, in one test 0.3 c.c. of

bile was added to a fatal dose of rat serum (1 c.c.) and, after standing 7 minutes, the mixture was injected, with only a very slight dyspnea resulting, but the test repeated at once with the same bile and serum caused death in 4 minutes. Two other tests gave similar results. The addition of cholesterin to toxic serum gave equally indefinite results.

Attempted Demonstration of Ferment Action.—Since it had been found that trypanosomes in contact with normal rat serum readily produced a very active anaphylatoxin, it was pertinent to inquire into the mechanism involved. It was quite natural to look on the process as enzymatic. The fact that heated trypanosomes, like heated proteins, starch, agar, etc., induce the same reaction, excluded the possibility of such ferment being derived from the organisms. The hypothetical ferment, therefore, must have its source in the normal serum, and, if it is brought into being by the presence of the trypanosomes, then it seemed reasonable to expect that a toxified serum should be capable of producing the same poison when diluted with one or more volumes of normal serum. When tests were made with this object in view, they apparently were successful and pointed to the existence of a ferment. Even a casual inspection of Tables 13 and 14 will reveal a suggestive resemblance to ferment action.

TABLE 13

APPARENT FERMENT ACTION: DILUTION OF TOXIC SERUM WITH NORMAL RAT SERUM
(1 + 1 SERIES)

Exper.	Guinea-Pig		Serum		Result of Injection of 1 c.c. of Test Dilution
	No.	Weight	Dilution	Incubation at 37 C.* (hr.)	
A	1	205	1:2	1	2'
	2	250	1:4	1	3'50"
	3	230	1:8	1	Slight
B	1	240	1:2	1	3'
	2	230	1:4	2	Severe
	3	250	1:8	4	2'
	4	215	1:16	4	7'
	5	218	1:32	8	Severe
	6	220	1:64	8	"
	7	250	1:128	16	6'
	8	230	1:256	24	Severe
	9	200	1:512	36	Very slight

* This indicates the time which elapsed between the consecutive tests; that for No. 1 of each set is from the start of incubation.

Table 13 consists of 2 distinct experiments A and B. For the first the blood of 10 nagana rats was defibrinated, pooled, and centrifugated at 3000 revolutions. The toxic serum was kept in a capped tube on ice for 8 days before use. A preliminary test showed that 1 c.c. was

fatal, whereas 0.75 and 0.5 c.c. produced only mild effects. Accordingly, 1 c.c. of this serum was added to 1 c.c. of normal rat serum, and this mixture, No. 1, was then placed at 37 C. for 1 hour; then 1 c.c. was taken out and tested. To the remaining half of Mixture 1, an equal volume of normal serum was added and this mixture, No. 2, was incubated and tested as before. In the same manner Mixture 3 was made and tested. It will be seen that Tests 1 and 2 resulted fatally; the latter representing a 1:4 dilution had but 0.25 c.c. of the original toxic serum.

Inasmuch as the third dilution failed to kill and the explanation seemed to be that not enough time had been allowed for the supposed ferment to act, the experiment was repeated, the digestion period being progressively increased. A portion of the same toxic serum used to start Exper. A was employed for Exper. B; the dilutions and tests were made consecutively as before. The results of this experiment confirmed and extended those obtained in the first trial. It will be seen from the table that even the seventh dilution of which the amount injected contained but $\frac{1}{128}$ c.c. of the original toxic serum, was fatal.

TABLE 14
APPARENT FERMENT ACTION: DILUTION OF TOXIC SERUM WITH NORMAL RAT SERUM
(1 + 3 SERIES)

Exper.	Guinea-Pig		Serum		Result of Injection of 1 c.c. of Test Dilution
	No	Weight	Dilution	Incubation at 37 C. (hr.)	
1B	1	200	1:4	4	4"
2B	2	188	"	2½	Very severe
3B	3	200	"	2	3'30"
3C	4	200	1:16	8	4'30"
3D	5	205	1:64	8½	3'10"
4B	6	175	1:4	3	3'10"
5B	7	230	"	3	4'15"
6B	8	175	"	2¾	3' 5"
7B	9	180	"	10	Moderate

The experiments of Table 13 were sufficiently suggestive to warrant further tests. These were made at different times during the following year, with a modified procedure. The toxic serum (1 c.c.) was diluted with 3 c.c. of pooled normal rat serum, giving a 1:4 dilution, usually designated as B. One cubic centimeter of B was diluted in like manner giving a 1:16 dilution, or C, and this process was continued to dilution D and even E. The advantage of this procedure is that the dilutions are made with the same pooled serum and that the mixtures are all incubated at the same time. Table 14 contains the results of tests

with several different B dilutions; in the case of Experiment 3, the dilutions B, C, and D were tested. In the last 4 experiments the toxic sera were each diluted with inactivated rat serum; for Tests 6 and 7 the serum was heated at 56 C. for one-half hour, while for 8 and 9 the sera were placed at 60 C. for the same time.

The results given in Table 13 and for the first 5 tests of Table 14 could be easily interpreted as showing the presence of a ferment. Thus, in Table 13, 1 c.c. of a 1:128 dilution killed, and 1 c.c. of the 1:256 dilution produced a severe shock; similarly, in Table 14 the 1:64 dilution was fatal. These results are, however, extremely fallacious and misleading, having an entirely different significance. In the first place normal rat serum on incubation may acquire a marked toxicity, and further this inherent change is accelerated by the presence of minute amounts of trypanosomes. Altho the toxic sera were centrifugated at 3000 revolutions, such minute quantities were not excluded. In similar work with agar even more striking results were obtained, as will be seen on reference to Part IV.

The last 4 tests in Table 14 have a direct bearing on this point, since different treated sera were diluted each with inactivated serum. The same toxic serum used in Test 2 was diluted with serum heated at 60 C. and used for Test 8. Another toxic serum was treated with 60 C. serum for Test 9. For Test 7 the washed trypanosomes were added to 3 c.c. of 56 C. serum and the mixture iced for 1 hour, then centrifugated; the serum thus treated was then diluted with 3 parts of the same inactivated serum and this on incubation became toxic. Test 6 was made at the same time, but in this case the organisms were mixed with normal serum, and after icing and centrifugation this treated serum was likewise diluted with the 56 C. serum. This dilution it will be seen was also toxic.

It would appear from these tests that inactivated serum is toxified about as readily as normal serum. The small number of tests, however, renders it unwise to place too much stress on these experiments. Indications have been obtained showing that agar may toxify inactivated serum (Part IV). In general, it is held that anaphylatoxin cannot be produced in heated sera, the only exception being the observations of Seitz⁹ and of Friedemann and Herzfeld,¹⁰ but in these instances the shock produced was not acutely fatal, death occurring after 1 or more hours. It may be mentioned in this connection that trypanosomes

⁹ Ztschr. f. Immunitätsf., 1912, 14, p. 91.

¹⁰ Centralbl. f. Bakteriöl., R., 1912, 54, Beiheft, p. 250.

which have been heated to 60 C. for half an hour, when added to normal rat serum and incubated, readily yield a toxic serum which in dose of 1 c.c. causes acute death.

It will be seen from what has been said that the activity of a ferment as regards anaphylatoxin-production is not established by these tests. The presence and action of such an agent are rendered still more doubtful by experiments made with sera centrifugated at very high speed (8000 revolutions).

Comparative Effect of High- and Low-Speed Centrifugation.—The work on agar anaphylatoxin, to be described in Part IV, first revealed the important fact that a treated serum apparently possessing a catalytic or ferment action, completely loses that property if centrifugated at a speed of 8000 revolutions for from 20 to 40 minutes. It seemed therefore that the catalytic action is not due to a substance in solution, but rather to something in suspension which is not fully removed by short centrifugation at low speed. To maintain the ferment idea it must be assumed that it is held loosely absorbed by the fine suspensoid.

In view of the results obtained with agar-treated serum it was desirable to see if this held true for the trypanosome serum. Four series of tests were made in which the comparative effect of centrifugation at 3000 and at 8000 revolutions was studied. The results were essentially identical with those obtained in the work with agar. It will be sufficient to describe 2 of these experiments.

For the tests given in Table 15, 7 heavily infected nagana rats were bled. In each case the heart pipet was instantly sealed with vaselin, plunged into ice water and the blood defibrinated. The operation of drawing and defibrinating the 7 bloods took 20 minutes. The bloods were then pooled in an iced flask and divided into 2 portions of 12 c.c. each, which were then placed in previously iced centrifuge tubes.

One portion was then centrifugated at 3000 revolutions for 7 minutes, at the end of which the corpuscles and the bulk of trypanosomes were thrown down. The supernatant serum was cloudy, however, and a rapid examination showed that this was due to the presence of organisms. The toxicity of 1 c.c. of this serum was tested at once, the balance being placed at 37 C. in the Roux water bath, and then tested at the intervals noted.

The centrifugation of the second portion, at 8000 revolutions for 40 minutes, was started at the same time as that of the first portion.

The water-clear serum thus obtained was tested at once, and the balance, placed at 37 C., was tested at the times shown in the table.

TABLE 15

I. COMPARISON OF EFFECTS OF DIFFERENT CENTRIFUGATION-RATES ON ANAPHYLATOXIN-PRODUCTION IN DEFIBRINATED BLOOD OF NAGANA RAT

Guinea-Pig		Serum			Result
No.	Weight	Centrifugation	Incubation at 37 C. (min.)	Intravenous Injection (c.c.)	
1	190	3000 r. p. m. for 7 min.	0	1.0	Nil
2	185		15	0.5	3/20"
3	185		19	0.25	3/45"
4	179		66	0.125	Severe
5	200	8000 r. p. m. for 40 min.	0	1.0	Very slight
6	203		15	"	Nil
7	205		30	"	Very slight
8	202		55	"	Nil

In this experiment special care was taken to check or prevent any action of the trypanosomes previous to the centrifugation. Hence, the precautions to keep the material cold. The time which elapsed from the beginning of the first bleeding to the injection of Guinea-pig 1 was 38 minutes; that to injection of Guinea-pig 5 was 86 minutes.

It will be seen from Table 15 that the cloudy serum, which was not toxic in dose of 1 c.c. when tested at once, on incubation rapidly became toxic. The lethal dose of 0.25 c.c. shows that the same degree of toxicity can be obtained with these organisms as with agar. By contrast, the clear serum was practically without effect when tested at once, or after incubation. The occasional twitches or slight jerky spasms were such as are often obtained with like amounts of normal serum (autoanaphylatoxin).

This experiment clearly shows that some trypanosomes must remain in the serum in order that it may become toxic. Their rapid and complete removal leaves the serum practically in a normal state.

The failure to kill with the clear serum cannot be explained by the supposition that the poison was either altered or thrown down by the prolonged high-speed centrifugation. Such a view would find no support in the next experiment (Table 16), and furthermore, it may be said that an actual test of a serum having a lethal dose of 0.25 c.c. showed that centrifugation at 8000 revolutions for 40 minutes was without effect on the toxicity.

As illustrative of the need of attention to details and speed, the following experiment is given (Table 16). It was made some days prior to the preceding one. Six infected rats were bled in 25 minutes; each heart pipet, after defibrination, was placed in ice water. The bloods were pooled and placed in cracked ice for 1 hour. The blood was then divided into 2 portions; one portion was centrifugated at 3000 revolutions for 4 minutes, while the other portion was swung at 8000 revolutions for 20 minutes. The first serum was very cloudy, because of the presence of some trypanosomes, while the second serum was perfectly clear. The time which elapsed from the first bleeding to the injection of No. 1 was 1 hour, 53 minutes; to injection of No. 5, 2 hours, 36 minutes.

TABLE 16

II. COMPARISON OF EFFECTS OF DIFFERENT CENTRIFUGATION-RATES ON ANAPHYLATOXIN-PRODUCTION IN DEFIBRINATED BLOOD OF NAGANA RAT

Guinea-Pig		Serum			Result
No.	Weight	Centrifugation	Incubation at 37 C. (min.)	Intravenous Injection (c.c.)	
1	180	3000 r. p. m. for 4 min.	0	1.0	5'30"
2	200		3	0.5	3'40"
3	185		12	0.25	Very severe
4	215		38	"	3'45"
5	200	8000 r. p. m. for 20 min.	0	1.0	3'25"
6	203		8	0.5	Very slight
7	210		30	"	Moderate
8	205		60	"	Severe

To the fact that the blood of each rat was defibrinated before icing, and to the further fact that the pooled blood, tho iced, was kept for an hour, is due the toxicity seen in Nos. 1 and 5. This means that in the second series the trypanosomes were removed after they had given rise to some poison. Unless a soluble ferment was present one would not expect an increase in the toxicity of this serum. There is, however, an apparent increase shown in Test 8, and this has also been noted in corresponding experiments with agar. It is more than likely that this effect is due to the animal's having a greater susceptibility. The same explanation holds true for the apparent increased toxicity of the serum in No. 4 as compared with No. 3. When duplicate tests are made variations of this kind are frequently met with (Table 45).

Maximal Toxicity.—The foregoing tests having shown that it was possible to obtain an anaphylatoxin in about 15 minutes such that 0.25 c.c. was lethal, it was desirable to see whether this result could be

obtained with reasonable frequency. With this object in view considerable effort was devoted to duplicating these results, especially that in Table 15, No. 3. The outcome of these tests, however, was most discouraging. Frequently, severe symptoms were obtained with 0.25 c.c. of the treated sera, but fatal results with that dose were extremely rare; in fact, only once, apart from the results tabulated, was this lethal dose obtained. In 12 of 24 tests it was possible to toxify the serum in 15 minutes at 37 C. so that 0.5 c.c. was fatal.

It is difficult to offer a satisfactory explanation of such failures, for without doubt several factors are concerned. The sera of animals unquestionably vary in the ease with which they can be toxified under apparently identical conditions. Furthermore, the proper amount of organisms or their state is a matter of moment just as it is in the case of agar. Again, as pointed out heretofore, single tests of toxicity are not a certain means of detecting the presence of a minimal lethal dose; a better indication is obtained when 2 guinea-pigs are injected at each period.

It is a very significant fact that the maximal toxicity is often reached within a few minutes. From the ferment standpoint one would expect a progressive increase, but such does not seem to be the case. Whether agar or trypanosomes are used as toxifying agents, the greatest toxicity, represented by a lethal dose of from 0.2 to 0.25 c.c., is frequently obtainable within 15 minutes or less, the incubation being at 37 C.

Apparent Loss of Toxicity.—In connection with the work on agar anaphylatoxin it was frequently found that the toxicity apparently rose and fell, thus indicating an oscillation or wave character. Similar tests made with trypanosome serum exhibited something of the same behavior. One of these experiments, made with guinea-pig serum, has already been described (Table 8, Chart 1); another test was made with rat serum. In view of the greater ease with which this serum can be toxified, it was possible to employ a much smaller dose (0.5 c.c.). The results are consequently the more instructive, as will be seen from Table 17 and Chart 2.

For this experiment, 6 heavily infected rats were bled in 20 minutes; the defibrinated pooled blood was at once centrifugated at 3000 revolutions. The trypanosome layer with the supernatant serum was then pipetted off, mixed, and placed in cracked ice for 1 hour. The only purpose of the latter step was the convenience of starting the tests

at that time. It is possible that icing has an unfavorable action on the toxicating power of the organisms. The mixture was then placed at 37 C.; and at each quarter-hour period 1 c.c. was removed, centrifugated, and 0.5 c.c. of the clear serum injected. As soon as the first fatal shock was obtained (No. 4), the entire mixture was centrifugated at 3000 revolutions* for 8 minutes and the resulting serum was then returned to the water bath. It follows therefore that the tests, beginning with No. 5, were made with serum from which the organisms had been removed; that is to say, the oscillations from this point on occur in a practically cell-free serum. Seemingly, the reaction when once initiated by the alien matter, in this case trypanosomes, continues even tho the original inciting cause be removed.

TABLE 17
APPARENT VARIATION IN TOXICITY OF RAT SERUM TREATED WITH T. BRUCEI

Guinea-Pig		Incubation of Serum at 37 C. (hr.)	Result of Intravenous Injection of 0.5 c.c.
No.	Weight		
1	182	$\frac{1}{4}$	Severe
2	183	$\frac{1}{2}$	Very severe
3	179	$\frac{3}{4}$	" "
4	200	1	3'25"
5	205	$1\frac{1}{4}$	Good
6	195	$1\frac{1}{2}$	3'50"
7	181	$1\frac{3}{4}$	3'55"
8	197	2	Slight
9	175	$2\frac{1}{4}$	3'50"
10	175	$2\frac{1}{2}$	5"
11	174	$2\frac{3}{4}$	7' 5"
12	179	3	3'45"
13	195	$3\frac{1}{4}$	3'20"
14	181	$3\frac{1}{2}$	Slight
15	176	$3\frac{3}{4}$	Very slight
16	173	4	Fair
17	171	$4\frac{1}{4}$	4'20"

The greater frequency of fatal results in this series as contrasted with that in which guinea-pig serum was used (Table 8) must be ascribed to a greater toxicity of the treated rat serum. The guinea-pig serum barely contained 1 lethal dose, whereas the rat serum probably contained nearly 2 lethal doses, since it has been shown that it can develop a titer of 0.25 c.c. The occasional failure to kill, or even to produce a severe shock, means not so much that the poison has decreased but rather that the resistance of the animal has increased (see Table 45). This resistance obviously becomes less marked when the serum injected contains several lethal doses. One thing which is perfectly clear is that the poison once made persists in the serum at 37 C. for at least 4 hours.

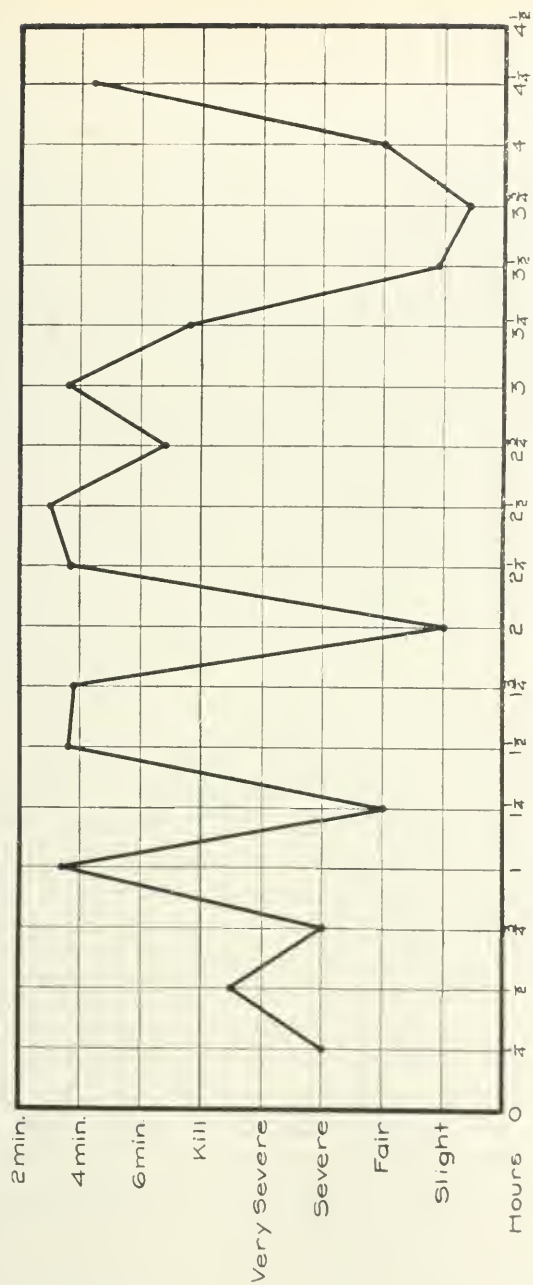


Chart 2. Apparent variation in toxicity of rat serum treated with *T. brucei* (Table 17).

Anaphylatoxin in Infected Rats.—Somewhat paradoxical is the fact that trypanosomes when incubated with normal serum readily yield a fatal poison, whereas the same organism in contact with the plasma of the living animal apparently does not do so. In the case of the very susceptible guinea-pig a very small amount of such poison if formed within the body ought to kill. With the rat the condition is otherwise, since it is extremely resistant to the poison. The phenomenon is not particularly difficult to understand from the standpoint of a physical reaction. It parallels perhaps the incoagulability of blood during life. The probable explanation is that while the organisms tend to change the physical state of certain plasma colloids, the enormous mass of other colloids in the plasma and in the cells exerts a check on the extent of this change. Certain it is, that when the infected blood is drawn into a test tube, the inhibitory action of the body mass is absent and hence the trypanosomes rapidly induce this change; in the absence of organisms the drawn blood passes through a similar reaction which finds its expression in the production of pre-coagulation toxicity (auto-anaphylatoxin), and in fibrin coagulation. While the organisms multiplying within the blood, or other parts of the body, are unable to produce an acute anaphylactic shock, they may bring about a gradual and moderate change represented perhaps by chronic intoxication and cachexia, seen especially in rabbits.

The studies on agar anaphylatoxin showed that the rat possesses a remarkable resistance to this poison. A dose sufficient to kill 100 guinea-pigs can be tolerated by the rat. This fact was first utilized to demonstrate the in-vivo production of anaphylatoxin in agar-shocked rats, and in the shock of specific anaphylaxis. It was eventually applied in determining whether or not this poison was present in the blood of heavily infected nagana rats. As a consequence of this natural resistance, such infected rats may well carry sufficient poison in their blood to kill several guinea-pigs. The rapid transfusion of a suitable amount of blood ought to establish the presence or absence of the poison.

For the following experiment a number of large rats, weighing about 180 gm., were inoculated with *T. brucei*. When the number of parasites in the blood had increased to about 100-150 per field, they were used for the tests. A syringe needle was introduced into the exposed heart, the desired amount of blood was drawn up and at once injected into the jugular vein of a guinea-pig.

It is essential for the success of this experiment that the transfer

time shall be as short as possible. A delay of a few seconds, especially with a large amount of blood, may result in a fatal shock even with normal blood, through the development of precoagulation anaphylatoxin. In Table 66, Part VI, will be found the results of transfusions of normal rat blood into guinea-pigs and these may serve as controls for this series. It will be seen that 4 c.c. of normal rat blood can be transfused with little or no effect, provided the transfer time is kept within about 45 seconds. Reference to Table 18 will show that this amount of nagana blood, with like transfer time, is fatal. This result would be more convincing if the transfer time was reduced to 20 or 30 seconds, a speed which is only possible with very large rats. It certainly would be of interest to repeat these tests with very large, almost moribund rats in which the maximal change could be expected.

As the tests stand, it may be conceded that a portion of the anaphylatoxin demonstrated by this method is made *in vivo*; a second portion by the trypanosome action within the syringe, and a third portion by precoagulation changes in the blood itself, likewise in the syringe. The sum of these three factors is expressed in the observed effects. It would not be logical to assume that all the anaphylatoxin present in the injected blood was formed within the rat.

TABLE 18
THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN RATS INFECTED WITH *T. BRUCEI*

Guinea-Pig		Intravenous Injection (c.c.)	Transfer Time* (sec.)	Result
No.	Weight			
1	180	2	30	Practically nil
2	183	"	33	Nil
3	190	3	37	Slight
4	215	4	51	3' 4"
5	215	"	40	4'14"

* By "transfer time" is meant the interval from the entrance of the syringe into the heart of the rat until its withdrawal from the vein of the guinea-pig (see Table 65).

Intravenous Injection of Washed Trypanosomes.—It has been pointed out that the trypanosomes in a heavily infected rat may give rise *in vivo* to some anaphylatoxin, but that in the case of the guinea-pig this must of necessity be less than 1 lethal dose, since otherwise acute death would follow. The mass of the body colloids must be looked upon as exerting a reversive action *pari passu* with the multiplication of the organisms, but in drawn blood this factor is removed and anaphylatoxin is rapidly formed. The sudden injection of a very large amount of trypanosomes might very well result in a disturbance of

equilibrium sufficient to bring on a typical anaphylactic shock. It is known that injections of bacteria, kaolin, and silicic acid can produce an acute fatal shock, and, as will be shown, the same is true for agar.

Six attempts were made to shock by this method. The washed organisms from 4 to 6 nagana rats were suspended in 1 c.c. of salt solution and injected into a guinea-pig of 200 gm. weight. In one such test marked prostration followed, the temperature in 20 minutes dropped to 34 C., and in 45 minutes it was below 29 C. Marked bleeding was present, indicating a noncoagulable blood; spasm appeared in about an hour and death occurred in 1 $\frac{1}{4}$ hours. In another test jerky spasms appeared in 3 minutes and continued for some time; the temperature in 20 minutes dropped to 35.5 C., but recovery took place. In the other tests no appreciable effect was observed.

Since subsequent experiments with injections of agar demonstrated the importance of a suitable dilution, it is highly probable that better results would have been obtained had the organisms been suspended in about 10 c.c. of salt solution or distilled water. The two results mentioned indicate that a 'trypanotoxin' action can be induced by the intravenous injection of washed trypanosomes into a guinea-pig. This action, as with so-called 'endotoxins' in general, does not mean that the cells are poisonous in the common acceptance of the term; they merely induce in vivo the same reaction as in vitro. This interpretation of endotoxin action will be made clear in connection with the agar work (Part VI).

Injection of Normal Sera into Nagana Guinea-Pigs.—Since trypanosomes react promptly with normal sera in vitro giving rise to anaphylatoxin, it was of interest to learn whether the injection of a normal serum into a heavily infected guinea-pig would bring on a similar reaction. Inasmuch as the guinea-pig serum does not give rise to a very active poison outside of the body it was hardly to be expected that it would do otherwise when injected into the infected animal. But with rat serum the result might well be different because of its capacity to yield a poison the lethal dose of which may be 0.25 c.c. or even less. With this object in view several tests were made with homologous serum and with the heterologous rat serum.

The guinea-pigs were tested on the 5th to the 10th day after inoculation; at that time they showed from 25 to 100 trypanosomes per field. The guinea-pig serum was injected in a dose of 5 c.c. No immediate effect was noted. In one animal convulsions were observed in 2 hours

and death occurred 6 hours after the injection. The fact that an untreated infected control died about the same time made it probable that the effect observed was not due to the injection. It should be added that this amount of guinea-pig serum may itself at times be fatally toxic.

In several of the treated animals there was, apparently, a few hours after the injection, an enormous increase in the number of parasites, and it seemed as if this was due to the serum injected. If this observation were true, it would be of value as a means of obtaining large amounts of parasites. Unfortunately, a number of attempts made to confirm this apparent multiplication were without result. The increase noted was merely such as is occasionally met with in the guinea-pig prior to death. It may be added that a somewhat similar observation on infected rabbits and guinea-pigs, subjected to anaphylactic shock, was reported by Scott.¹¹

Toxicity of Normal Rat Serum.—The injection of rat serum into infected guinea-pigs gave more promising results. Thus, while in the first trial, 5.3 c.c. of normal rat serum were injected with no noticeable effect other than a rapid increase in the number of parasites, in a second test, 5 c.c. produced a typical shock and death in 2 minutes; while in a third, the same result was produced by 4 c.c.

These tests were made at a time when it was believed that normal rat serum was not toxic in dose of 6 or 7 c.c. The death could not be ascribed to specific anaphylaxis, because the guinea-pigs had been inoculated with a strain maintained in these animals. It seemed therefore that the object of the experiment had been realized, showing that the trypanosomes could react in vivo with the injected rat serum. In due course of time, however, it was found that normal rat serum could be toxic in the doses mentioned, and this fact made it probable that the observed deaths were due directly to the injected serum.

The experiment was therefore repeated on two different occasions. In one of these tests the infected guinea-pig received 4 c.c. without any effect. In a second test, an infected animal after receiving 4 c.c. of a pooled serum died of typical shock in 3½ minutes; but 2 controls which were given 3 and 4 c.c., respectively, of the same pooled serum likewise died in 3½ and 4½ minutes!

It is evident therefore that normal rat serum may, at times, cause typical anaphylactic shock and death in dose of 3 or 4 c.c. It may be added that this pooled serum was derived from 8 rats. The blood as

¹¹ Bull. Soc. path. exot., 1911, 4, p. 674.

fast as removed from one was discharged into a cylinder provided with a rod. The operation for the lot took half an hour. The combined blood was then thoroughly whipped, centrifugated, and the resultant serum iced for about 6 hours before use. The remarkable toxicity of this serum might have been due to the delayed mass defibrination; it might also have been due to some one of the rats having a chance infection with *T. lewisi*. Unfortunately, no examination was made to exclude the latter possibility. If the blood was from normal animals, then the fatal dose of 3 c.c. represents the smallest lethal dose of normal rat serum.

It may be concluded from the foregoing that the trypanosomes within the body of a guinea-pig cannot toxify an injected serum. Apparently, the condition (body mass) which restrains the production of anaphylatoxin in the plasma of the infected animal, also exerts a like control over an alien serum.

SUMMARY

Anaphylatoxin was produced by 5 different trypanosomes, including the nonpathogenic *T. lewisi*.

It is made not only by the living cells, but also by the dead, more or less autolyzed, cells, and even by such when heated to 60 C.

The same mass of trypanosomes can be used repeatedly to toxify different lots of serum without apparently any limit to their inducing power. This serial production in one experiment extending over 8 days, was carried through 20 tests with no indication that the organisms were weaker than at the beginning.

Rat serum is preferable to guinea-pig serum since it yields a poison which may be 12 or more times as active as that obtained with the latter; the respective lethal doses being 0.25 c.c. of the former and 3 of the latter.

The speed of poison-production under favorable conditions is very rapid and quickly reaches a maximum, the poison then persisting for a long time. Thus, infected defibrinated rat blood or serum, when incubated for 1 or 2 minutes, may become fatally toxic. With an incubation of about 15 minutes, it is possible to produce a toxic serum such that 0.25 c.c. will cause acute anaphylactic death.

Sera inactivated at 56 C. or 60 C. for half an hour apparently can be toxified.

The anaphylatoxin was found to persist at 37 C. for more than 4 hours; at about 0 C. it may persist for an indefinite time. Thus, a

surra serum was still toxic after icing for 171 days; a nagana serum was likewise active for 35 days. Attempts at detoxifying by addition of normal serum, bile, or cholesterin resulted negatively.

Toxic sera can induce toxicity in normal sera, the mixtures representing a high dilution of the former. This result is not due to the action of a ferment, but to minute amounts of trypanosomes still present in the inciting serum; or, in the case of very prolonged incubation, to the production of autoanaphylatoxin.

A comparison of the effects of different centrifugation-rates on chilled, rapidly defibrinated blood shows that sera obtained at slow speed readily become toxic; the sera secured at a very high speed do not.

Transfusion experiments indicate the possible presence of anaphylatoxin in heavily infected rats. The formation of this poison in corpore may lead to sudden deaths or to chronic intoxication or cachexia.

The injection of normal rat serum into infected guinea-pigs does not result in the in-vivo production of poison; any effect observed is due to the primary toxicity or autoanaphylatoxin of such injected serum.

The toxicity of trypanosomal sera, when tested at regular intervals, appears to show more or less oscillation. Similar variations are met with in tests with anaphylatoxins produced by other agents, and even in normal sera. They are not due to changes in the amount of poison, but to varying resistance of the test animals.

The injection of large amounts of washed trypanosomes into guinea-pigs may result in the production of anaphylatoxin in corpore. The effects are not due directly to the organisms, but to the disturbance in the colloidal state of plasma constituents, caused by the alien material.

The 'trypanotoxins' are therefore disturbers of equilibrium; the result is a poison-production in vivo as well as in vitro. The mode of action of endotoxins in general will be found to be of the same nature.

The participation of a ferment in this reaction is contraindicated by the speed of production, the rapid attainment of maximal level, the behavior of inactivated sera, and by the results of centrifugation.

The syndrome of symptoms and autopsy findings consequent on the injection of the trypanosome anaphylatoxin are those of the intoxication of true anaphylaxis. The two poisonings are to be considered as identical.

II. AGAR ANAPHYLATOXIN: GUINEA-PIG SERUM

F. G. NOVY AND P. H. DEKRUIF

SYNOPSIS

INTRODUCTION

INCONSTANCY OF RESULTS

METHODS

EXPERIMENTS WITH AGAR SOL (50 C.)

EXPERIMENTS WITH AGAR GEL (0 C.)

EXPERIMENTS WITH SOL-GEL AGAR

CENTRIFUGATION OF SOL-GEL MIXTURES

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

SUMMARY

The previous study on trypanosome anaphylatoxin showed clearly that the serum was the real source of the poison, and that the organisms merely played a part common to all so-called in-vitro antigens. And, since the collection of the trypanosomes was a laborious as well as expensive procedure, it was desirable to employ a more convenient reagent for the further prosecution of the study. The agar solution of Bordet¹ proved to be a most useful reagent and was employed, therefore, in the greater part of the work that followed. The serum of choice was that of the rat, on account of the ease with which it gives rise to a very active anaphylatoxin; but the sera of the rabbit and guinea-pig were utilized, from time to time, to confirm and extend the observations made with rat serum.

Bordet prepared his solution as follows: 0.5 gm. of agar was dissolved in 100 c.c. of 0.75% salt solution and this then was tubed and sterilized. The next day the gel was broken up by thorough shaking, and 5 volumes of fresh guinea-pig serum were added to 1 volume of the agar. The mixture (0.2 + 1), after being kept for 2 hours at 37 C. was centrifugated. The clear liquid when injected intravenously into guinea-pigs in dose of 4 to 5 c.c. gave the characteristic symptoms of anaphylactic poisoning with death in a few minutes. These results were soon confirmed; among others, by Nathan² and by Tchernoroutzky,³ who found that the serum could be toxified by one-tenth the amount of agar employed by Bordet, that is, 0.02 c.c. of agar (= 0.1 mg. agar) per cubic centimeter of serum. It will be shown that considerably less than the amount mentioned will produce anaphylatoxin, especially with rat serum.

Inconstancy in results was a striking feature in the work of others, and such was likewise found to be the case in this study. For that

¹ Compt. rend. Soc. de biol., 1913, 74, pp. 225, 877.

² Ztschr. f. Immunitätsf., 1913, 17, p. 478.

³ Compt. rend. Soc. de biol., 1913, 74, p. 1213.

reason, an enormous amount of time was devoted to ascertaining the optimal conditions necessary to the maximal and speediest production of poison. The understanding of a reaction is possible when the result of chance is replaced by one of certainty. Most of this explorative work was done with rat serum, and a considerable portion of it with guinea-pig serum. It may be stated here that eventually it became possible to toxify rat serum in less than 15 minutes, and to such an extent that 0.25 c.c. caused acute death.

Jobling and Petersen,⁴ by the action of chloroform on guinea-pig serum, obtained a toxic serum which was fatal to a 210-gm. guinea-pig in dose of 0.31 c.c. Bronfenbrenner,⁵ by bringing together placenta and pregnant guinea-pig serum, secured an anaphylatoxin, the lethal dose of which was 0.5 c.c. per 260 gm. of guinea-pig. In view of these results, it seemed that it should be feasible, by means of agar, to toxify guinea-pig serum to the same degree. This, however, was not accomplished, tho on a number of occasions the lethal dose was reduced to 1 c.c. and even approximated 0.75 c.c. Without doubt, further effort will give an even more toxic serum. As the result stands, it means that the guinea-pig serum possesses some inhibiting factor which does not exist in that of the rat. Strikingly suggestive is the fact that the guinea-pig, which is looked upon as being extremely sensitive to anaphylatoxin, should yield a relatively difficultly toxifiable serum, whereas the insusceptible rat gives a serum which can be rendered toxic with the greatest ease.

The best method of treating serum with agar which has been devised is liable to give somewhat inconstant results. Tchernoroutzky believed that the serum of young guinea-pigs, weighing from 400 to 500 gm., became more poisonous than that from the adult. He placed the lethal dose of the anaphylatoxin from the former at 1.2 c.c. per 100 gm. (ranging from 0.5 to 2 c.c.); while for that having the latter origin, the dose was from 2 to 3 c.c. (ranging from 1.5 to 4.3 c.c.). It has not been possible to confirm this view; the serum from adult guinea-pigs can yield as active a poison as that from the relatively young. Irregularity in results occurs in both kinds of sera. Trifling details in manipulation often give considerable variations in results.

The behavior of pregnant serum with placenta, as noted by Bronfenbrenner, might suggest that it was more labile than that of the male, but tests that were made with this in mind, failed to show any difference. Thus, by the sol-gel method, which will be considered later, a mixture of sol and pregnant serum (in the ratio of 0.1:1), after being iced for 18 hours and then kept at 37 C. for 21 hours, caused acute death in dose of 3 c.c., while a dose of 2 c.c. was without effect. In other tests, the mixture of gel and pregnant or nonpregnant serum (0.25:1) was incubated for 1 hour, then centrifugated at 3000 revolutions, after which it was again placed at 37 C. for 3 and 35 hours, and tested; the treated sera in dose of 2 c.c. were without effect.

Another suggested explanation of the irregularity in anaphylatoxin-production deals with the age of the serum, it being supposed that a serum which has been kept for a day or more is less toxifiable than a fresh one. This view cannot account for the inconstant results in these studies since the sera were usually employed immediately after centrifugation, or at most 2 or 3 hours later. More-

⁴ Jour. Exper. Med., 1914, 19, p. 485.

⁵ Ibid., 1915, 21, p. 480.

over, it is a fact that sera which have been iced for a day can be toxified. Thus, Exper. 1 of Table 19 shows the production of poison in a 36-hour serum. Like results have been obtained with rabbit sera, and in the case of rat serum kept for 1 day there is no difficulty in producing the maximal toxicity of a 0.25-c.c. lethal dose.

A review of the results obtained, not only with guinea-pig serum but also with that of the rat and rabbit, shows clearly that the sera of a given species are subject to variation, and that no perfect regularity in toxifying with agar can be expected. Wherein the individuality of the serum rests cannot be indicated at this time. It certainly is not directly associated with the presence of lipoids, since repeatedly it was found that a white milky serum was as readily toxifiable as one that was water-clear. Tests made with fasting sera likewise gave results not unlike those with ordinary sera.

As regards the speed of anaphylatoxin-production in guinea-pig serum, under the influence of agar, it may be said that with proper conditions a minute or two suffices to render a large dose of serum acutely fatal. No special effort was made to establish this point, but in one instance (Table 30, No. 1) a dose of 3 c.c. was fatal within 8 minutes after the agar-serum mixture was made. In another instance, 3 c.c. was fatal after incubation for 10 minutes. Indeed; this dose tested after incubation at 37 C. for 15 minutes is regularly toxic. Tests made on 5 different days, the incubation being 30 minutes, gave toxic sera such that 1.5 c.c. was acutely fatal. Similarly, in 3 experiments, 1 c.c. was found to be the lethal dose after an incubation of from 1½ to 2 hours.

The agar solution employed in these studies was prepared by the addition of 100 c.c. of distilled water to 0.5 gm. of ordinary thread agar. The mixture, in a plugged Erlenmeyer flask of 150-c.c. capacity, was usually sterilized by heating in an autoclave at 120 C. for 5 minutes. Sterilization at 100, 110, 120, or 130 C. seems to have no effect on the toxifying power of the agar, and yet it is possible that the hydrolytic cleavage, by prolonged heating at higher temperatures, may affect the physical state thereby altering the inducing power. Nathan,⁶ it may be mentioned, obtained about the same result with starch boiled for 2 hours or one-half minute. On the other hand, with inulin⁷ he found that while the suspension toxified, the solution obtained by warming to 68 C. for 2 minutes was without effect.

The agar prepared as here described, on cooling solidified to a solid gel. This was measured out for tests by means of a cut-off full-bore pipet, either as a solid, or a semigel, after thorough shaking of the solid mass. The solidity of a gel will vary with the extent of the supporting sides of the container. Thus, in a 150-c.c. Erlenmeyer flask the agar will form a gel so solid that its surface will not show

⁶ Ztschr. f. Immunitätsf., 1913, 18, p. 646.

⁷ Nathan, *ibid.*, 1914, 23, p. 209.

the slightest tendency to drop when the flask is placed horizontally; on the other hand, in a 500-c.c. flask the same amount of agar will form only a thin layer on the bottom which will not hold up when the flask is tilted. As a rule the agar was transferred to a smaller flask (50-c.c.), which was kept nearly full. Before use, the agar was always liquefied by heating in a water bath at 100 C. for 15 minutes. It was then placed either in a Roux water bath at 37 C., or at 50 C., for 2 hours to form a sol; or in cracked ice for 1 or 2 hours to form a hard gel.

The consistency of an agar undoubtedly exerts a considerable influence on its action as a toxifying agent. One ideal condition is that the agar should be distributed in the serum as homogeneously and in as fine a state of division as possible. Obviously, this condition is hardly realized when the solid or semi-solid gel is added directly to the serum. On the whole, better and more consistent results were obtained when the agar was measured out as a sol. As will be shown, the sol-serum mixture when incubated at once does not give as good results as when incubated after a period of icing.

The mixture of agar and normal guinea-pig serum yields a fair precipitate on incubation, a fact recognized by Bordet. He also noted that this precipitate did not occur in serum which is inactivated at 56 C. In normal rabbit serum the separation is less marked, while in the rat serum it is decided. With rat serum this is considerably decreased when the serum is heated to 50 C. for half an hour. The quantity of precipitate which forms is a fair index of the capacity of the serum to yield anaphylatoxin. The precipitate is not to be interpreted as due to an adsorption process whereby a preformed poison is liberated or unmasked; or to one whereby a ferment is uncovered and allowed to act on the serum proteins, thus giving rise to poisonous cleavage products.

For other details of the technic employed reference must be made to Part I.

EXPERIMENTS WITH AGAR SOL AT 50 C.

The tests with agar sol were made with the expectation that the greater fluidity would give an extreme dispersion of the agar throughout the serum, thereby securing a maximal toxifying power. In these and similar tests with other sera it is to be borne in mind that the agar remains dispersed as a sol. The relatively feeble action of the dispersed sol as contrasted with that of the gel will be apparent when comparison is made with the subsequent work.

The results as seen from Table 19 were not particularly encouraging. The irregularity in the poison-production was especially perplexing, and the cause was not understood until later. The chief fallacy in this and much of the early work lay in the failure to make serial injections, at short intervals, immediately after mixing the sol and serum. Moreover, the individual resistance of the recipient must also be remembered. The real value, of course, lies in the positive tests which indicated the presence of a lethal dose of the poison; the failure to kill must not be given too rigid an interpretation.

For the 9 experiments recorded in Table 19, a 50 C. sol was prepared in the manner indicated heretofore. This was added to guinea-pig serum, previously warmed to 37 C., in the ratio of 1:4, the amounts used being usually 3 and 12 c.c.

respectively. The mixture was made in a small Erlenmeyer flask, and to secure thorough distribution it was swung for a definite time. This was 1 minute for Expers. 1 to 6; 2 minutes for Exper. 9, and $\frac{1}{4}$ and $\frac{1}{2}$ minute respectively for Expers. 7 and 8. Each mixture was then centrifugated at 3000 revolutions for 5 minutes as soon as made, or after a short incubation at 37 C. (Expers. 3 to 6). In a few tests the centrifugated serum was injected at once (Nos. 1, 13, 15, 19); in these cases the interval between mixing and injection was about 10 minutes. In all the other tests, the treated centrifugated serum was incubated for 2 hours or more, at 37 C. The amount of serum actually injected is given in the table, allowance being made for the increase in volume due to the water left after centrifugation.

The serum employed for Exper. 1 had been iced for nearly 36 hours. It will be seen that this old serum gave as good results as the fresh sera. A pooled serum was used for Expers. 2 to 6, another pool served for Expers. 7 and 8, and still another for Exper. 9. The same agar, on different days, was used for Expers. 1 to 6; it was sterilized at 120 C., while that used for Expers. 7 to 9 was heated at 130 C.

It will be seen that, under the conditions given, a toxic dose of 3 c.c. was obtained in 6 of 9 experiments, usually after incubation for 2 hours. In one test, 1.7 c.c. proved fatal.

TABLE 19
ACTION OF AGAR SOL (50 C.) ON GUINEA-PIG SERUM (37 C.) (RATIO 0.25:1)

Exper.	Guinea-Pig		Serum*			Result
	No.	Weight	B. C. at 37 C.	A. C. at 37 C. (hr.)	c.c. (intra-venously)	
I	1	195	—	—	3.0	Very slight
	2	190	—	2½	"	2'45"
II	3	170	—	2	"	3'50"
	4	170	—	20½	1.7	Very slight
III	5	172	2'	2	3.0	Good
	6	225	"	6	"	Slight
IV	7	170	5'	2	"	Fair
	8	172	"	5	"	4'45"
V	9	170	10'	2	"	Fair
	10	185	"	6	"	2'50"
VI	11	175	15'	2	"	Fair
	12	225	"	6	"	Fair
VII	13	175	—	—	"	Slight
	14	195	—	2	"	Fair
VIII	15	200	—	—	"	Very slight
	16	202	—	2	"	3'20"
	17	176	—	"	1.7	3'
	18	170	—	"	0.85	Slight
IX	19	230	—	—	3.0	11 hr.
	20	170	—	2	"	4'25"
	21	175	—	"	1.7	Very slight

* The dash indicates that the mixture was centrifugated as soon as made. B. C. and A. C. mean before and after centrifugation, respectively.

The 10 experiments given in Table 20 differ from the preceding (Table 19), in that the serum was chilled to 0 C. before addition of the sol. The mixtures were swung for 1 minute and then placed in cracked ice for varying periods of time, after which they were centrifugated; the resulting treated serum was then placed at 37 C. for 2 hours and injected. In these tests 2 pooled sera were used, one for Expers. 3 to 8, and the other for the remaining. The same agar was used for all the trials.

TABLE 20

ACTION OF AGAR SOL (50 C.) ON GUINEA-PIG SERUM (0 C.) (RATIO 0.25:1. MIXTURE KEPT AT 0 C., THEN CENTRIFUGATED AND INCUBATED)

Exper.	Guinea-Pig		Serum			Result
	No.	Weight	B. C. at 0 C.	A. C. at 37 C. (hr.)	c.c. (intravenously)	
I	1	197	—	2	3	5/10"
II	2	220	1'	"	"	Near-kill
	3	171	1'	"	"	Slight
III	4	200	2.5'	"	"	3/40"
	5	170	2.5'	"	2	Severe
IV	6	170	5'	"	3	Fair
V	7	207	10'	"	"	Slight
VI	8	215	15'	"	"	Very slight
VII	9	195	30'	"	"	Near-kill
VIII	10	190	1 hr.	"	"	Near-kill
IX	11	187	2 hr.	"	"	Fair
X	12	170	3	"	"	Very slight

The results obtained in this series of tests were decidedly inferior to those of the series recorded in Table 19; in only 2 of 10 experiments was the serum rendered toxic so that the lethal dose was 3 c.c., tho 3 tests gave 'near-kills.' An essential condition, not provided in this series, is incubation at 37 C. prior to centrifugation. The effect of such incubation is indicated in Table 19; in the experiments there recorded the sol-serum mixture had an initial temperature of 37 C. or better (see also Table 22).

The series of tests was made to see the effect of icing for variable periods of time, and it would seem from the table as if the short period gave the better result. Here again is a fallacy of interpretation, since extremely severe shocks were obtained in Tests 2, 9, and 10. Had duplicate tests been made, that is, 2 guinea-pigs inoculated at the same time with the same material, the number of deaths without-doubt would have been increased.

The conclusion which can be drawn from these two sets of experiments is that the 50 C. sol can be used to toxify serum, but the results are not so good as with other methods. Further, incubation of the sol-serum mixture is quite essential. A 37 C. sol was not tried with guinea-pig serum since a greater effect was expected with that of 50 C. As it is, the lethal dose of 3 c.c. represents the toxifying power under these conditions.

EXPERIMENTS WITH AGAR GEL, 0 C.

In the endeavor to ascertain the best conditions for anaphylatoxin-production, many experiments were made with solidified agar or gel.

With the object of having this in as definite a state as possible, the agar for each experiment was first liquefied in a water bath, at 100 C. for 15 minutes, and then placed in cracked ice at 0 C. for 1 hour. Before use, this hard gel was broken up by vigorous shaking for about 20 seconds and the semisolid mass was then measured out by means of a full-bore pipet. In other tests, this cut-off pipet was plunged into the solid gel to the desired depth, and the cylinder of definite volume was then transferred to the serum flask.

The serum employed in these tests was also iced for at least half an hour. After the addition of the gel, the flask was vigorously swung for about 2 minutes in order to break up the agar as finely as possible. A homogeneous mixture under these conditions is out of the question; an examination will always show the agar to be in lumps of varying size, up to 2-3 mm. in diameter.

The mixtures of gel and serum, both at 0 C., were then treated in various ways. For the experiments given in Table 21, the mixture was kept at 0 C. for a given length of time, after which it was centrifugated at 3000 revolutions; in Expts. 2, 3, and 4, it was kept for 2, 6, and 12 minutes respectively; in the others, 15 minutes. The same agar and a pooled serum were used for the 3 experiments mentioned; for Expts. 1 and 5 another pooled serum and agar were used. The clear serum was then tested for its toxicity either at once, or after incubation at 37 C. for the stated time.

The results it will be noted were rather poor since but 2 fatal shocks were obtained in the 6 experiments. As in the case of the results given in Table 20, this failure must be ascribed in part to the lack of incubation of the gel-serum at 37 C. prior to the centrifugation; and, in part, to the infrequent testing. The reaction induced by the agar occurs best at higher temperatures. If, therefore, the agar is removed by centrifugation before this reaction takes place, obviously no toxicity other than the primary should be observed. However, complete removal of the agar does not occur with ordinary centrifugation and hence some reaction occurs on subsequent incubation, as will be seen from this and other tables.

In another series of experiments, similar to that just detailed, the gel-serum mixtures were kept at 0 C. for 6, 12, 24, 36, and 72 hours, respectively. Each mixture was then centrifugated at 3000 revolutions for 4 minutes, incubated, and tested at varying intervals from 1 to 32 hours. The test dose was 1.5 c.c. or less in all these experiments. Three typical acute deaths were obtained; one of these occurred with the mixture which had been iced for 6 hours and then, after centrifugation, incubated for 12 hours; with the set iced for 36 hours, one death resulted at the 6-hour test, and one at the 9-hour test, the latter being due to 1.25 c.c. of serum. Three other guinea-pigs showed severe effects but in the majority (19 of 25) the result was slight or nil. It may be added that in one other experiment similar to this one, the gel-serum mixture being iced for 21 hours, centrifugated, and then incubated for 14 hours, a fatal acute shock was obtained with 1.25 c.c.

TABLE 21

ACTION OF AGAR GEL (0 C.) ON GUINEA-PIG SERUM (0 C.); (RATIO 0.25:1. MIXTURE KEPT AT 0 C., THEN CENTRIFUGATED AND INCUBATED)

Exper.	Guinea-Pig		Serum			Result
	No.	Weight	B. C. at 0 C. (hr.)	A. C. at 37 C. (hr.)	e.c. (intra- venously)	
I	1	178	$\frac{1}{2}$	—	3	Very slight
	2	170	$\frac{1}{2}$	$6\frac{1}{2}$	"	Slight
II	3	180	1	$3\frac{1}{2}$	"	Very severe
	4	198	"	9	"	Very slight
III	5	170	"	$3\frac{1}{2}$	"	$3'50''$
	6	180	"	9	"	Slight
IV	7	186	"	$3\frac{1}{2}$	"	Very slight
	8	185	"	9	"	Slight
V	9	185	$1\frac{1}{2}$	—	"	Very slight
	10	205	$1\frac{1}{2}$	$5\frac{1}{2}$	"	Slight
VI	11	210	3	—	"	Very slight
	12	170	3	6	"	$3'30''$

These results, as pointed out, are to be ascribed to the minute amounts of residual agar left in the serum after centrifugation. It is always possible, however, that the mere incubation of normal guinea-pig serum extending over a long period of time, with tests at frequent intervals, might show increased primary toxicity. Such increase has been frequently met with in the case of rat serum.

An attempt at the complete removal of the agar was made in the following experiments. In these the gel-serum mixtures were kept at

0 C. for 1, 3, 6, 9, 12, 18, and 24 hours, then centrifugated at 8000 revolutions for 20 minutes, after which they were placed at 37 C. and tested at 3-, 9-, and 18-hour periods, the test dose being 3 c.c. It was expected that the high speed of centrifugation would remove all of the agar and, as a result, little or no poison would be formed. The tests which were made with the mixtures iced for 1 and 3 hours were strictly negative. Those made with mixtures which were iced for 6 and 9 hours caused acute death after incubation for 9 hours. Likewise, fatal shocks were obtained with the mixtures which had been iced for 12 and 24 hours, the tests being made after incubation for 18 hours. The mixtures which had been iced for 18 hours produced very severe shocks when tested at the 9- and 18-hour periods. It would seem that in the gel-serum mixtures iced for 6 hours or more a change was started similar to that at 37 C., but weaker. Still, the continued action of traces of agar is possible notwithstanding the high speed of centrifugation. Hence, the 4 deaths in 21 tests can be ascribed to either one of these causes.

TABLE 22

ACTION OF AGAR GEL (0 C.) ON GUINEA-PIG SERUM (0 C.) (RATIO 0.25:1. MIXTURE KEPT AT 37 C. THEN CENTRIFUGATED AND REINCUBATED)

Exper.	Guinea-Pig		Serum			Result
	No.	Weight	B. C. at 37 C.	A. C. at 37 C. (hr.)	c.c. (intra-venously)	
I	1	195	—	2	3.0	Very slight
	2	220	—	5½	"	"
II	3	176	2¼'	2	"	"
	4	218	2¼'	5	"	5'35"
III	5	195	5'	2	"	Severe
	6	223	5'	5	"	Slight
IV	7	195	10'	2	"	2'40"
	8	195	10'	5	1.5	Very slight
V	9	201	15'	2	3.0	"
	10	215	"	5	"	"
VI	11	215	"	2	"	Very severe
	12	171	"	4	"	2'40"
VII	13	206	30'	—	"	Very slight
	14	212	"	2	"	3'55"
	15	195	"	5	2.0	Fair
VIII	16	185	1 hr.	—	3.0	3'5"
	17	222	"	2	2.0	Slight
	18	170	"	4	2.0	3'15"
	19	175	"	4	1.0	Very-near-kill
IX	20	175	2 hr.	—	3.0	3'55"
	21	185	"	2	3.0	3'40"
	22	183	"	5	2.0	3'15"
	23	170	"	5	1.0	Slight

By way of contrast with the foregoing, it is in order to present another series of experiments. The essential difference between the two sets lies in the fact that the gel-serum mixtures were placed directly at 37 C. for a variable time, after which they were centrifugated at 3000 revolutions for 10 minutes, and then the clear sera were again incubated for the periods stated in Table 22. It will be seen that the contact of agar and serum, at 37 C., before centrifugation, is a necessary factor to poison-production.

The series of 9 experiments given in Table 22 were made on 2 consecutive days. A pooled serum was used for Experiments 1 to 5, and another for Experiments 7 to 9. Experiment 6 was a duplicate of Experiment 5, but was made with a different serum.

It would seem from this series of experiments that incubation of the gel-serum mixture at 37 C. for 1 or 2 hours before centrifugation was the best treatment. It might be of interest to have made serial injections at intervals of 15 minutes with such material, and especially with such as had been kept at 37 C. for a longer time, 4 hours or more. Under such circumstances it might be possible to toxify the serum so that 1 c.c. would be a lethal dose. It will be seen from the table that 2 c.c. was fatal in Experiments 8 and 9, and that 1 c.c. produced a 'very-near-kill' in No. 19.

EXPERIMENTS WITH SOL-GEL AGAR

This method of treatment was first developed in connection with the work on rat serum, in which it proved to be superior to other procedures. Consequently, it was desirable to apply it to guinea-pig serum. Here also it gave exceptional results, as will be seen from Tables 24 and 26. The method undoubtedly offers the best possible conditions as regards the uniformity of a mixture, tho even here it must not be forgotten that the amount of shaking given a mixture influences the degree of sol division and hence the result.

The procedure consists first in liquefying the agar in a water bath at 100 C. for 15 minutes, after which it is placed in a Roux bath at 37 C. for 2 hours. In passing, it may be added that at this temperature the agar remains in the sol state for a day or more. It is then added to the pooled serum, previously warmed to 37 C., and the mixture is thoroughly shaken for 1 minute. This is then packed in cracked ice, usually for 2 or 3 hours, tho a shorter time is perhaps equally good. The finely subdivided agar is thus brought into a gel state, tho the mixture, depending on the amount of agar, may remain perfectly fluid. This finely dispersed gel on subsequent incubation rapidly induces the toxifying action in the ambient serum.

The toxicity of such mixtures was tested at given intervals. When the amount of agar is very small, the uncentrifugated fluid can be injected directly. With larger amounts of agar it is best to centrifugate a small portion. It has seemed on several occasions as if the presence of agar in the fluid injected modified the toxicity, rendering it less marked.

For most purposes the sol was measured out directly. When very small amounts were desired a 10-fold dilution with distilled water (37 C.) was made. This, on further dilution with 1 or 9 parts of water, gave dilutions such that 0.1 c.c. corresponded to 0.005 and 0.001 c.c. of sol, respectively. This insured a minimal dilution of the serum to be tested.

The volume of the guinea-pig serum used in each series of tests was not less than 10 c.c. and often from 20 to 30 c.c. A series of mixtures, each containing a definite amount of sol per cubic centimeter of serum, was tested. For convenience of reference these mixtures, designated by numbers, are presented in tabular form indicating the amounts of sol and of agar present in each, per cubic centimeter of serum, as well as the ratio of dry agar to serum.

TABLE 23
THE CONCENTRATION OF DIFFERENT AGAR-SERUM MIXTURES

Mixture	Sol (c.c. per c.c. of Serum)	Agar (mg.)	Agar-Serum Ratio 1:
1	0.0005	0.0025	400,000
1a	0.001	0.005	200,000
2	0.0025	0.0125	80,000
3	0.005	0.025	40,000
4	0.025	0.125	8,000
5	0.05	0.25	4,000
6	0.1	0.5	2,000
7	0.25	1.25	800
8	0.5	2.5	400
9	1.0	5.0	200

For the following tests the injection dose was placed at 1.5 c.c., or less, in order to get at the maximal poison-production. It is hardly necessary to state that with 3 c.c. the acutely fatal shock would have been more frequent. Indeed, it is possible, that with such dose even Mixture 2 might be found to toxify, and this would certainly be the case if the large dose (5 c.c.) of Bordet was used.

Mixture 1.—This mixture was kept in cracked ice for 3 hours, then at 37 C.; tests were made at intervals of 1½ hours up to 24 hours. In about one-half of these tests no effect was observable. In the others, slight excitability, deep respiration, and few jerky spasms were noted. In the tests made at 13½ and 19½ hours, dyspnea and spasms were well defined.

It will be shown that a corresponding mixture with rat serum, on incubation for 12 hours, killed in dose of 1 c.c. This fact indicates that even guinea-pig serum may show positive evidence of toxification provided larger doses be injected. Table 23 shows that this mixture represents an agar-serum ratio of 1:400,000.

Mixture 2.—This series of tests was made at the same time as the preceding, and under like conditions. The effects were about the same as those noted; the most marked results being at $1\frac{1}{2}$, $13\frac{1}{2}$, and $19\frac{1}{2}$ hours.

Mixture 3.—One experiment similar to the preceding with tests at $1\frac{1}{2}$ -hour intervals up to 12 hours, gave very severe shocks (dyspnea, spasms, convulsions) at $4\frac{1}{2}$, $7\frac{1}{2}$, and 9 hours.

In another experiment, tests being made at half-hour intervals up to $3\frac{1}{2}$ hours, a 'near-kill' was obtained in 1 hour; the other tests were relatively mild. In the belief that if the tests were made at quarter-hour intervals a fatal result would be obtained, another set was tested, in this way, up to 2 hours, but the effect in all was mild; possibly a more reactive serum would have given the desired result.

Additional tests were made with similar mixtures kept in ice for 1, 6, 9, and 14 hours, respectively, before incubation. In each of these experiments only 2 injections were made, at 12 and at 18 or 24 hours. Here also but mild effects were noted. In view of the variable resistance of guinea-pigs to the poison, occasional tests of this kind have very little meaning, when one is operating with minimal lethal doses; as pointed out, heretofore, the better procedure is to make the tests in duplicate, at frequent intervals.

It would appear from the foregoing that this mixture, which represents an agar-serum ratio of 1:40,000, is almost sufficient to toxify so that 1.5 c.c. will be fatal. In view of this, and of the further fact that rabbit serum is toxified by this mixture, there can be no doubt but that a dose of 3 c.c. would have given some fatal shocks. Since the agar-serum mixture of Bordet (1 + 5) has 1 mg. agar per cubic centimeter (1:1000), it will be seen that the one used in these tests contains but one-fortieth as much.

In a single trial of a corresponding gel-agar mixture, kept at 37 C. for 2 hours, Nathan obtained slight effects, the dose injected being 3.5 c.c. It is of interest by way of comparison, to note that Mixture 3 tested with rat serum, toxified it in 15 minutes so that 0.5 c.c. was fatal; while in 1 hour, 0.25 c.c. produced a like result.

Mixture 4.—Several of the experiments made with this mixture showed marked poison-production. Preliminary trials were made with mixtures iced for 1, 3, 6, 9, and 24 hours, respectively, before incubation. In each of these experiments only 2 tests were made, at 12 and at 18 or 24 hours. The results were slight or nil, except with the mix-

ture which was iced for 6 hours. This, incubated for 12 hours, killed in dose of 1.5 c.c. and at the 24-hour test, in dose of 1 c.c., it gave a very severe shock or near-kill. This result led to a more extended series of tests with another mixture iced at 0 C. for 6 hours; these, which are given in Table 25, show that, after incubation for $1\frac{3}{4}$ hours, a dose of 1.5 c.c. is fatal and that 1 c.c. is likewise at the 2-hour test.

Two additional experiments were made, the mixture in each case being iced for 3 hours. The first of these, incubated at 37 C. and tested at half-hour intervals up to 12 hours, was made at the same time as the first experiment described under Mixture 3; the same pooled serum was used for the two experiments. The tests made at $1\frac{1}{2}$, 3, and $4\frac{1}{2}$ hours gave very severe shocks; those at 6 and $10\frac{1}{2}$ hours caused acute death; while at 12 hours, 1 c.c. produced a violent shock. The other tests were relatively mild. The second experiment, given in Table 24, shows that a dose of 1.5 c.c. was fatal after only 1 hour's incubation. The results of this experiment should be compared with the corresponding experiment with Mixture 5, made at the same time and with the same pooled serum.

TABLE 24

ACTION OF SOL-GEL ON GUINEA-PIG SERUM (MIXTURE 4, RATIO 0.025:1, KEPT AT 0 C. FOR 3 HR., THEN INCUBATED AND CENTRIFUGED)

Guinea-Pig		Serum		Result
No.	Weight	B. C. at 37 C. (hr.)	c.c. (intravenously)	
1	180	$\frac{1}{2}$	1.5	Good shock
2	170	1	"	3'30"
3	183	$1\frac{1}{2}$	1.0	Very slight
4	171	2	"	Slight
5	202	$2\frac{1}{2}$	1.5	Very slight
6	175	3	"	3'30"
7	180	$3\frac{1}{2}$	"	Nil
8	185	5	"	3'40"

The results of two experiments with Mixture 4, iced for 3 and 6 hours, respectively, are given in Tables 24 and 25. It will be noted that the speed of poison-production under the conditions given, is fairly rapid. Further that it is possible to produce a 1-c.c. lethal dose with guinea-pig serum. The agar-serum ratio in this mixture is 1:8000, or one-eighth that employed by Bordet; and, moreover, the toxicity is increased 4- and even 5-fold, the dose employed by him being 4-5 c.c.

Mixture 5.—In general, the results obtained with this mixture were about the same as those with the preceding one. It was used not only for the experiments which immediately follow, but also for others to

be described later (Tables 27, 28, 29, *et al.*). This and the next are probably the most useful mixtures.

Here as in the two preceding series, experiments were made with mixtures which had been iced for 1, 3, 6, 9, and 14 hours, respectively, before incubation. The first of these, kept at 0 C. for 1 hour, was incubated and tested at 3, 12, 18, and 24 hours. The first injection produced but slight effect, the second a severe shock, the third a near-kill, while the fourth had only moderate effect. Another mixture, likewise kept at 0 C. for 3 hours and then incubated, proved fatal when tested at 13 hours; retested at once in dose of 1 c.c. it caused a severe shock. This dose tried again at 18 hours caused a delayed death, in 7 hours.

TABLE 25

ACTION OF SOL-GEL ON GUINEA-PIG SERUM (MIXTURE 4, RATIO 0.025:1, KEPT AT 0. C. FOR 6 HR., THEN INCUBATED AND CENTRIFUGED)

Guinea-Pig		Serum		Result
No.	Weight	B. C. at 37 C. (hr.)	c.c. (intravenously)	
1	200	$\frac{1}{2}$	1.5	Very slight
2	197	1	"	Nil
3	177	$1\frac{1}{2}$	"	Severe quiet shock
4	176	$1\frac{3}{4}$	"	2:55"
5	170	2	1.0	3:55"
6	170	$2\frac{1}{4}$	0.5	Very slight
7	175	$2\frac{1}{4}$	1.0	Severe
8	195	$2\frac{1}{2}$	1.5	3:20"
9	180	3	"	3:45"
10	207	$3\frac{1}{2}$	"	Slight
11	206	4	"	Very slight
12	209	$4\frac{1}{2}$	"	Slight
13	180	5	"	Near-kill
14	195	$5\frac{1}{2}$	"	Slight
15	182	6	"	Severe
16	180	$6\frac{1}{2}$	"	Slight

In 3 other experiments in which the mixtures were kept at 0 C. for 6, 9, and 14 hours, respectively, and then incubated and tested at 12 and again at 18 or 24 hours, only slight effects were obtained. Apparently, prolonged icing is not as favorable for this mixture as is the shorter treatment.

Of three other mixtures, kept at 0 C. for 3 hours, one (designated No. 6) was tested after incubation for 1, 3, and 9 hours. The first injection resulted in a severe, the second in a fair, and the third in a very severe, shock. The experiment was repeated (No. 7) with another like mixture, the injections, however, being made at $1\frac{1}{2}$ -hour intervals up to $7\frac{1}{2}$ hours. The injection at $1\frac{1}{2}$ hours gave a fair shock, at 3 hours a near-kill, and at $4\frac{1}{2}$ hours a typical acute death; the other tests were

slight or nil. In the next attempt (No. 8), made with a like mixture, the tests were tried at half-hour intervals up to $3\frac{1}{2}$ hours. This experiment was made at the same time and with the same pooled serum as was the experiment recorded in Table 24; the results were nearly alike in the two series, except that the effects with this mixture were less; the test at $1\frac{1}{2}$ hours proved fatal, that at 3 hours gave a near-kill, while the others were slight or nil.

On reference to Tables 27 and 28 it will be seen that this mixture can render guinea-pig serum toxic in dose of 3 c.c. in 10 or 15 minutes.

Mixture 6.—Here, as in the preceding, a number of mixtures were tested after being kept at 0 C. for 3 hours. One of these tested after incubation for 1, 3, and 9 hours gave but slight effects compared with those of the corresponding experiment (No. 6) with Mixture 5, which was made at the same time and with the same pooled serum.

A second experiment was made at the same time and with the same pooled serum as in No. 7 of the preceding series. After incubation for $1\frac{1}{2}$ hours the mixture killed in dose of 1.5 c.c., while 1 c.c. produced a severe shock. The same result was obtained at 3 hours, but at $4\frac{1}{2}$ hours there was practically no effect.

In a third experiment in which the tests were made at intervals of 15 minutes up to $1\frac{1}{2}$ hours, that at one-half hour killed, and those at 1 and $1\frac{1}{2}$ hours produced severe shocks, while the other tests gave but slight effects.

In a fourth experiment, in which the mixture was kept under liquid paraffin, a very severe shock was obtained in 1 hour and a kill at 3 hours. Another mixture, after incubation for one-half hour, in dose of 3 c.c. caused acute death; retested at 1 hour, it killed in dose of 1.5 c.c.

Mixture 7.—Three experiments were made with this mixture. In each case it was kept at 0 C. for 3 hours, and then incubated. The first of these corresponded to the third of the preceding series and was made at the same time and with the same pooled serum. The test with 1.5 c.c. at 1 hour killed; with 1 c.c. a very severe shock was obtained at $1\frac{1}{4}$ hours, and at $1\frac{1}{2}$ hours it killed.

In the second experiment, 1.5 c.c. was fatal in the test at one-half hour; at 1 hour, 1 c.c. caused a very severe shock, but had slight effect at $1\frac{1}{2}$ hours. In a third experiment, the mixture after incubation was injected directly without previous centrifugation. The injections made at one-half hour intervals had little or no effect, until at 3 hours, when 1.5 c.c. caused typical death.

Mixture 8.—Three experiments with this mixture were made. In each the mixture was iced for 3 hours, then incubated, and at intervals 2.25 c.c. were removed and centrifugated; the resulting fluid, representing 1.5 c.c. of serum, was then injected. In one experiment (No. 1) 7 consecutive tests made during the first 2 hours, at 37 C., gave little or no effect. This result should be compared with that of the first experiment made with Mixture 9. The two series were made at the same time, side by side, with the same pooled serum. The failure in this case, if not due to a slight difference in the shaking of the mixtures, must be ascribed to individual variation in the animals tested. The experiment was therefore repeated, the mixture this time being given a more vigorous shaking for 1 minute; in this trial the test made at one-half hour caused typical death in 7 minutes, while 4 succeeding tests, up to 2 hours, gave but slight effects.

In a third experiment, the injection made after incubation for one-half hour was acutely fatal. The injection of 1 c.c. at the 1-hour period gave a very severe shock, but a like amount at 1½ and 2 hours had little effect.

It will be seen, therefore, that with this mixture also it is possible to toxify the serum so that 1.5 c.c. will kill. Nathan,² with corresponding amount of agar, failed to produce death with a 3-c.c. dose; this fact and the irregularities occurring with the ordinary mixture (0.2 + 1) led him to believe that an excess of agar was unfavorable. It is true that a mixture having much agar, at times does seem to give poor results, but that is probably not due merely to the presence of a large amount of agar. The experiments with this mixture and especially with the one that follows show that serum can be toxified under these conditions.

Mixture 9.—This mixture consisted of equal parts of sol and serum (Table 23). As in the preceding, the mixtures were iced for 3 hours and then incubated. At intervals 3 c.c. were removed and centrifugated; the resulting fluid, corresponding to 1.5 c.c. of serum, was then injected. In one experiment, corresponding to No. 1 with Mixture 8, and made at the same time and with the same pooled serum, the injection of 1.5 c.c. at ½- and 1½-hour periods caused death in 3 and 4 minutes, respectively, whereas the other 5 tests up to 2 hours were practically negative.

In a second trial which was made at the same time and with the same pooled serum as No. 2 with Mixture 8, the first test, made after

15 minutes at 37 C., gave a good shock; tests made at $\frac{1}{2}$, $\frac{3}{4}$, 1, and $1\frac{1}{2}$ hours showed little or no result, whereas at 2 hours 1.5 c.c. and also 1 c.c. produced typical acute fatal shocks.

Summation.—In the series of experiments with the 9 mixtures, as just outlined, the test dose only once exceeded 1.5 c.c.; occasionally, it was reduced to 1 c.c. On account of the smallness of the dose it is to be expected that the majority of the injections would yield little or no effect, and that the number of fatal shocks would be small. With such small doses the individual resistance of the animals is a factor in the scarcity of positive results.

Several important facts are to be noted. In the first place, it is seen that the toxicity appears to develop early and to persist for many hours. Further, that it is possible to produce in normal guinea-pig serum a toxicity such that 1.5 and even 1 c.c. are fatal. Also, that very minute amounts, as well as very large amounts of agar, may bring about the poison-production. Likewise that the sol-gel method is superior to the others in the speed and extent of the reaction induced. With very minute amounts of agar apparently a longer incubation is required than with larger quantities.

The results are collated in the following table:

TABLE 26
SUMMATION OF RESULTS WITH THE SERIES OF SOL-GEL MIXTURES; THE OCCURRENCE OF FATAL SHOCKS WITH 1 AND 1.5 C.C. OF TREATED SERUM

Mixture	Number of Tests	Fatal Shocks with 1 c.c.	Fatal Shocks with 1.5 c.c.
1	17	0	0
2	17	0	0
3	36	0	0
4	46	1; at 2 hr.	9; at 1, $1\frac{1}{4}$, $2\frac{1}{2}$, 3, 5, 6, $10\frac{1}{2}$, and $12\frac{1}{2}$ hr.
5	30	1; at 18 hr.	3; at $1\frac{1}{2}$, $4\frac{1}{2}$, $13\frac{1}{2}$ hr.
6	19	0	3; at $\frac{1}{2}$, $1\frac{1}{2}$, 3 hr.
7	28	1; at $1\frac{1}{2}$ hr.	3; at $\frac{1}{2}$, 1, 3 hr.
8	20	0	2; at $\frac{1}{2}$, $\frac{1}{2}$ hr.
9	14	1; at 2 hr.	3; at $\frac{1}{2}$, $1\frac{1}{2}$, 2 hr.

CENTRIFUGATION OF SOL-GEL MIXTURES

The effect of centrifugation at 8000 revolutions for 20 minutes on gel-serum mixtures which had been kept at 0 C. for varying periods from 1 to 24 hours, as regards poison-production has been briefly touched upon (p. 543). Inasmuch as only two or three injections, at long intervals, were made in those experiments, it was desirable to repeat the 3-hour one, employing, however, a sol-gel mixture, a longer centrifugation, and more frequent tests. The immediate object was to

ascertain whether the mere contact of agar with serum, at 0 C., was sufficient to induce a change such that on subsequent incubation poison would be produced. To secure an unequivocal answer to a query of this kind, the agar must be completely removed from the serum, otherwise the presence of a mere trace, acting for a long time, may be sufficient to toxify. This removal of agar cannot be accomplished fully by centrifugation at 3000 revolutions, and for that reason such centrifugated sera do show poison-production on incubation. The only feasible way to effect a complete removal seemed to be by high-speed centrifugation for a very long time. Accordingly, a speed of 8000 revolutions for 60 minutes was resorted to.

The following experiment may be considered as a crucial test of the question as to the action of agar in mixtures iced for 3 hours, tho it was not made all in one day and with one pool of serum. On each of 3 consecutive days, a sol-gel mixture (No. 5) was prepared in the usual way, then immediately placed in cracked ice for 3 hours, after which it was centrifugated at the speed and for the time just mentioned. The perfectly clear pooled serum of each day was then transferred to test-tubes, which were placed in the Roux bath at 37 C. The injections were made at half-hour intervals up to and including the 10½-hour period. The injection dose contained the equivalent of 3 c.c. of serum. The total number of tests was 21, and of these Nos. 1 to 3 were made on the first day, Nos. 4 to 11 on the second, and Nos. 12 to 21 on the third.

The results of this experiment were practically nil; occasionally a few slight jerks, restlessness, but no dyspnea. It follows therefore that under these conditions, notwithstanding the large dose of serum and the risk of developing an autotoxic state by long incubation, there is no increase in toxicity beyond that which is present or developed in untreated normal serum. This would appear to be contrary to what might be expected if the reaction were purely one of adsorption, since after being iced for 3 hours the mixture might be expected to give some evidence of the latter.

The experiments hitherto given made it clear that, in order to secure a production of poison by the agar, heat must be applied before subjecting the mixture to centrifugation. This point established, the next step was to ascertain the effect of the complete removal of agar from a sol-gel mixture which had been kept at 0 C. for 3 hours and then incubated for a short time at 37 C.

For this purpose a sol-gel mixture (No. 5) was prepared in exactly the same manner as before, the amounts used being 2.5 c.c. of sol and 50 c.c. of guinea-pig serum. After thorough shaking for 1 minute the mixture was iced for 3 hours, and then placed at 37 C. for 10 minutes. The bulk of the mixture was now centrifugated at 8000 revolutions for 1 hour; a small portion was swung at the same time at 3000 revolutions for 6 minutes, and the equivalent of 3 c.c. of serum injected at once into Guinea-pig 1, producing a typical fatal shock. It is seen therefore that the mere incubation of the sol-gel mixture for 10 minutes was sufficient to render it toxic in dose of 3 c.c.; and further, that the bulk which was subjected to the high-speed centrifugation was initially toxic.

TABLE 27
CENTRIFUGATION AT 8000 R.P.M. APPLIED AFTER INCUBATION AT 37 C. FOR 10 MIN.
SOL-GEL-MIXTURE 5, RATIO 0.05:1

Guinea-Pig		Serum			Result
No.	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	c.c. (intra- venously)	
1	212	10	—	3	3'10"
2	210	—	0	"	Fair shock
3	208	—	$\frac{1}{2}$	"	Very slight
4	195	—	1	"	Slight
5	190	—	$1\frac{1}{2}$	"	Nil
6	210	—	2	"	4'
7	200	—	$2\frac{1}{2}$	"	5'
8	198	—	3	"	Fair
9	175	—	$3\frac{1}{2}$	"	2'55"
10	185	—	4	"	Good
11	190	—	$4\frac{1}{2}$	"	4'10"

After centrifugation at 8000 revolutions, the clear serum was placed at 37 C. and tested at once, and at half-hour intervals, as indicated in Table 27. It will be seen that immediately after centrifugation the serum was apparently less poisonous than before; that after incubation for 2 hours the perfectly clear serum again became toxic, and that this state persisted, seemingly in wave form, till the end. This experiment should be compared with the corresponding one with rabbit serum, in which tests were made at quarter-hour intervals (Table 35). The results are expressed graphically in Chart 3. As pointed out heretofore, this and similar curves while conceivably due in part to variations in the amount of the poison must not be read wholly in that sense; for variation in the resistance of the animals must be considered, and probably this is the main if not sole cause of the wave character.

In the foregoing experiment the high-speed centrifugation was started at the time when the sol-gel mixture was toxic, that is, at what

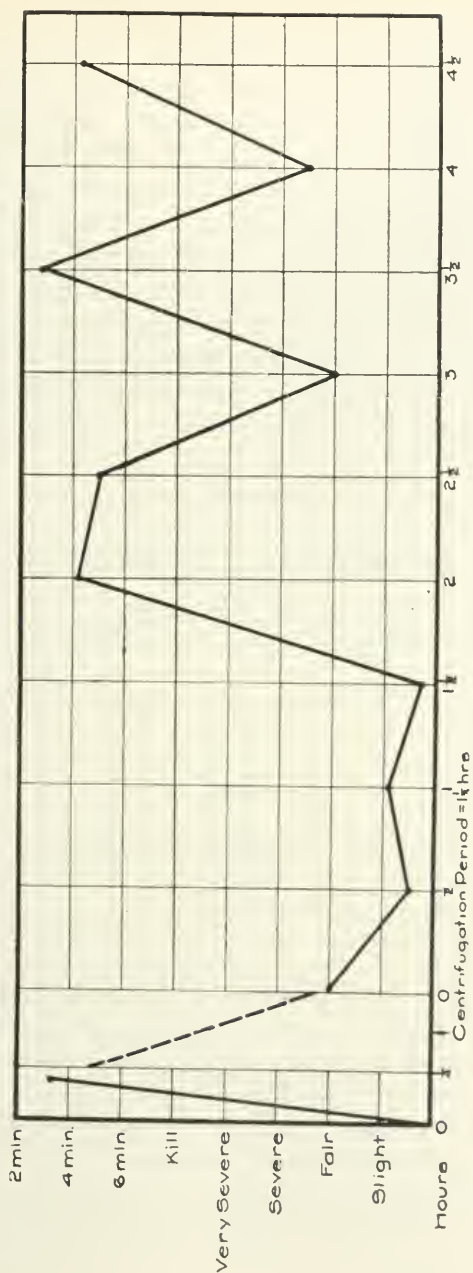


Chart 3. Centrifugation of the sol-gel mixture at 8000 r.p.m. after incubation. Apparent variation in toxicity of treated guinea-pig serum (Table 27).

might be called the crest of the toxic wave. It seemed that a different result might be obtained if the centrifugation was begun when the initial toxicity had dropped, and accordingly the experiment was repeated on the following day. The preparation of the sol-gel mixture and icing were exactly the same as before. The mixture was then placed at 37 C. and, at intervals as shown in Table 28, portions were removed, centrifugated at 3000 revolutions for 4 minutes and injected. It will be seen that incubation for 5 and 10 minutes seemed to produce but little poison, whereas incubation at 15 minutes gave rise to a fatal anaphylatoxin. The expected drop in toxicity did not occur until after incubation for 1 hour. At this point, the balance of the mixture was centrifugated at 8000 revolutions for 1 hour; the resulting clear serum was transferred to the Roux bath at 37 C., tests being made at once, and at half-hour intervals.

TABLE 28
I. CENTRIFUGATION AT 8000 R.P.M. APPLIED AT THE FIRST DROP IN TOXICITY
SOL-GEL-MIXTURE 5, RATIO 0.05:1

Guinea-Pig		Serum			Result
No.	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	c.c. (intra- venously)	
1	175	5	—	3	Slight
2	180	10	—	"	Slight
3	175	15	—	"	3'25"
4	170	30	—	"	3'30"
5	172	45	—	"	4'10"
6	204	60	—	"	Very slight
7	185	—	0	"	3'5"
8	200	—	1/2	"	Very severe
9	212	—	1	"	Very slight
10	185	—	1 1/2	"	3'30"
11	200	—	2	"	4'
12	210	—	2 1/2	"	2'50"
13	197	—	3	"	3'40"
14	212	—	3 1/2	"	3'30"
15	198	—	4	"	3'10"

It will be seen from Table 28 and the corresponding chart (4), that the toxicity which seemed to have returned at the close of the centrifugation, was absent in the next 2 tests, after which it reappeared and remained in evidence until the end, giving rise to 6 consecutive kills. A similar toxic plateau is indicated in Chart 3, and also in Chart 5. It is to be expected that incubation of the sol-gel mixture for 1 hour would give a greater toxicity than incubation for only 10 minutes at 37 C., as in Table 27; and consequently a greater number of deaths. It may be questioned, however, whether this alone accounts for the peculiar curve.

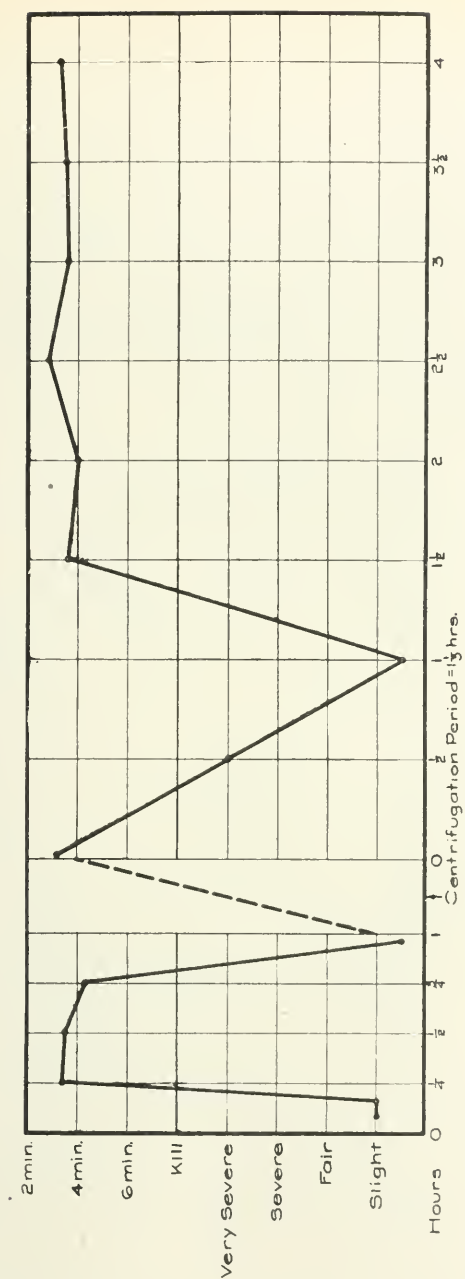


Chart 4. Centrifugation of the sol-gel mixture, at 8000 r.p.m.; after incubation till first drop in toxicity occurs. Apparent variation in toxicity of treated guinea-pig serum (Table 28).

The striking result obtained in the preceding experiment made its repetition desirable. This was done on the following day, the conditions being the same as before. In this trial the first kill was obtained at the 30-minute period, tho the 2 preceding tests gave severe shocks. The apparent drop in toxicity occurred at 1 hour, the same as before. The mixture was then centrifugated at 8000 revolutions for 1 hour, and the clear serum was put at 37 C., tests being made as usual at once and at half-hour intervals. Here again, after centrifugation, a rise in toxicity was seemingly obtained, followed in 1 hour by a marked decrease, and then a return to a plateau, which remained to the end, through 7 consecutive tests. The results of this experiment are given in Table 29, and again in Chart 5.

TABLE 29
II. CENTRIFUGATION AT 8000 R.P.M. APPLIED AT THE FIRST DROP IN TOXICITY
SOL-GEL-MIXTURE 5, RATIO 0.05:1

Guinea-Pig		Serum			Result
No.	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	c.c. (intra- venously)	
1	210	10	—	3	Severe shock
2	184	15	—	"	Very severe
3	194	30	—	"	3'10"
4	206	45	—	"	3'30"
5	208	60	—	"	Very slight
6	197	—	0	"	Severe
7	196	—	1/2	"	Very severe
8	209	—	1	"	Very slight
9	190	—	1 1/2	"	4'
10	190	—	2	"	3'40"
11	187	—	2 1/2	"	Near-kill
12	206	—	3	"	Very-near-kill
13	190	—	3 1/2	"	2'50"
14	173	—	4	"	Very-near-kill
15	198	—	4 1/2	"	3'30"

It is hardly to be expected that the same curve characteristics of toxicity should be met with in every experiment of this type. Apart from irregularities in technic, some variation must occur unless it be assumed that the pooled guinea-pig serum is chemically and physically a constant, and further that the animals are all equally susceptible. Thus, somewhat different results were encountered in an experiment intended to duplicate that given in Table 27. The pooled serum in this case was obtained from the blood of old female guinea-pigs, some of which weighed over 1 kilo. The sol-gel mixture 5, after being iced for 3 hours and incubated at 37 C. for 20 minutes, was centrifugated at 8000 revolutions for 1 hour. The serum was then tested at once and the balance, placed at 37 C., was tested at half-hour intervals (13 tests).

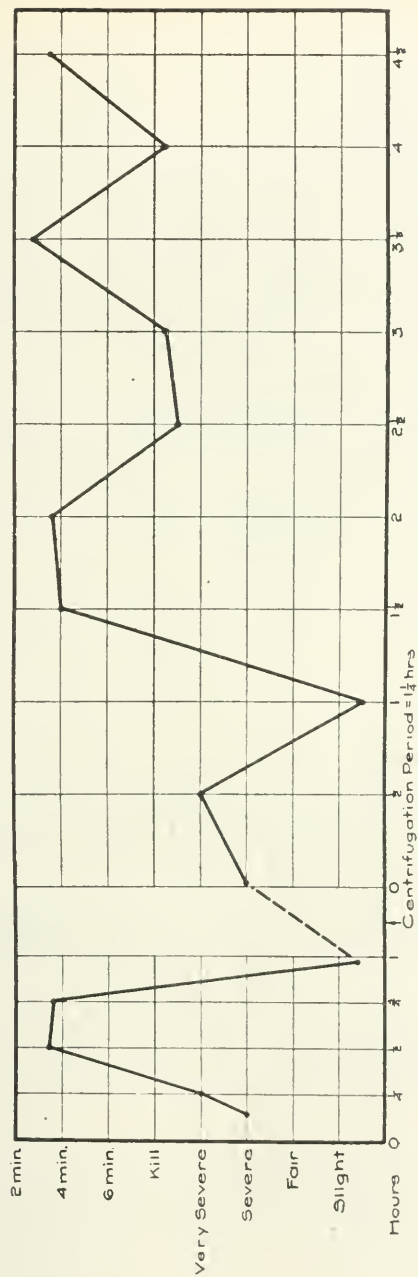


Chart 5. Conditions similar to those of Chart 4; see Table 29

The test which was made at once after centrifugation killed, as also did those made at $\frac{1}{2}$, $1\frac{1}{2}$, and 6 hours. Very severe shocks were obtained at the 2- and 4-hour periods, while the other tests had but slight effects. This series of tests if charted would show no indication of a plateau; the simplest meaning is that the amount of poison produced was less than in the other experiments and hence the individual resistance of the animals is more in evidence.

Summation.—These experiments with sol-gel Mixture 5, which had an agar-serum ratio of 1:4000, show that guinea-pig serum can be toxified in a dose of 3 c.c. on incubation for 10 minutes or more. The poison once produced remains in the serum for hours, even after high-speed centrifugation. Apparent oscillations in the poison content are seen, but these are to be interpreted as due to variations in the test animals. As in the case of trypanosomes, heat must be applied to the agar-serum mixtures. The failure of sera to become toxic after high-speed centrifugation of iced unheated mixtures indicates that adsorption plays no part in the production of poison. Further, ferment activity is not indicated, since there is no increase in the toxicity of incubated sera after high-speed centrifugation of agar-serum mixtures which have been iced and incubated for a short time.

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

The possible presence of a ferment, in some way activated by the agar, was suggested here as in the previous work with trypanosomes. It seemed as if the hypothetical enzyme, once set free, should be demonstrable by means of serial dilutions into a pooled normal serum. On the other hand, if the poison existed preformed in the serum, and was merely uncovered by the removal of an antagonistic body, as supposed by Bordet¹ and others, then such solutions should show no increase in toxicity. A considerable number of experiments were made with rat and guinea-pig serum; those with the former will be considered later. The results at first were interpreted as indicative of a ferment action, but further study showed that such conclusions were not justifiable.

It will be of interest to present 2 such experiments, in which the conditions were practically identical. A pooled guinea-pig serum was employed for each series of tests, and the same agar was used for both. Three cubic centimeters of the 50 C. agar sol were added to 12 c.c. of the serum, previously warmed to 37 C.; the mixture, after thorough

agitation for 1 minute, was at once centrifugated at 3000 revolutions for 5 minutes. The resulting fluid designated as A was divided into 3 portions: one was tested at once for its toxicity; the second was placed at 37 C. and tested at intervals as noted in the tables; the third was used to make dilutions. For the latter, 2 c.c. of A were added to 6 c.c. of pooled serum, giving a 1:4 dilution, or B. Diluting 2 c.c. of B in like manner gave the 1:16 dilution, or C, and this process was repeated yielding the 1:64 or D dilution, and the 1:256 or E dilution. These dilutions, in small Erlenmeyer flasks, were put in a Roux bath at 37 C. and tested at the times indicated.

TABLE 30

I. APPARENT FERMENT ACTION: PROGRESSIVE DILUTION OF 'TREATED' GUINEA-PIG SERUM WITH NORMAL SERUM

Series	Dilution	Guinea-Pig		Serum		Result
		No.	Weight	c.c.(intra-venously)	Hours at 37 C.	
A	—	1	182	3	0*	6'30"
	—	2	195	3	4	3'15"
	—	3	185	2	4	Good shock
B	1:4	4	175	3	2	30'
	1:4	5	185	"	4	Good shock
C	1:16	6	190	"	4	Good shock
	1:16	7	201	"	23	45'
D	1:64	8	203	"	"	3'40"
E	1:256	9	180	"	"	4'20"
Control		10	202	"	"	9½ hr.

* This test was made 8 minutes after mixing the agar and serum; the mixture was not incubated.

The results of these tests are given in Tables 30 and 31. It will be seen in Table 30 that Serum A was acutely fatal at the initial test, which was made 8 minutes after mixing the sol and serum. It is to be noted that the control tests in both experiments with same serum, incubated for a long time but otherwise untreated, resulted in very delayed deaths. Similar delayed deaths occurred with dilutions C, D, and E of Table 31, and are to be interpreted as instances of primary or autotoxicity.

In the foregoing experiments the mixture was made with 50 C. sol and 37 C. serum, and consequently a favorable temperature for interaction was maintained for some minutes. Other tests, made under like conditions, gave equally good results. In fact in a number of experiments, the A and B portions were fatal when tested after incubation

for 2 hours at 37 C. On the other hand, mixtures of iced gel and serum, kept in ice for $\frac{1}{2}$, $1\frac{1}{2}$, and 3 hours, and then centrifugated without any previous incubation at 37 C., yielded a serum which usually did not become toxic on subsequent incubation, and moreover failed to induce poison-production when diluted with normal guinea-pig serum.

In interpreting these results and those given in Tables 30 and 31, it is necessary to bear in mind that guinea-pig serum possesses an initial or primary toxicity which persists and may even increase on incubation. An even more important consideration is the fact that centrifugation at 3000 revolutions does not remove all the suspended agar. The presence of residual agar after such centrifugation can be easily shown by recentrifugating the apparently clear serum at 8000 revolutions, when a distinct coating of agar will be noted on the bottom of the glass.

TABLE 31

II. APPARENT FERMENT ACTION: PROGRESSIVE DILUTION OF 'TREATED' GUINEA-PIG SERUM WITH NORMAL SERUM

Series	Dilution	Guinea-Pig		Serum		Result
		No.	Weight	c.c. (Intra-venously)	Hours at 37 C.	
A	—	1	217	3	0	Slight
	—	2	197	"	6	Severe
	—	3	170	"	18	4 hr.
B	1:4	4	180	"	6	Good
	1:4	5	170	"	18	3/20"
C	1:16	6	177	"	6	Slight
	1:16	7	192	"	18	5 hr.
D	1:64	8	200	"	30	Good
	1:64	9	180	"	48	4 hr.
E	1:256	10	170	"	48	7½ hr.
Control		11	185	"	48	12 hr.

This residual agar is carried over into the successive dilutions, tho of course in decreasing amount, and the toxicity induced by such traces of agar, plus that which is primarily present, readily accounts for the observed results. This summation effect is most marked in the B dilution, for in 3 of 6 other tests acutely fatal sera were obtained in about 2 hours. When the B dilution was made into rat serum, the result was even more marked, for 0.5 c.c. of such would become acutely fatal. This phase of the subject will receive further attention in connection with rat serum in Part IV, where it will be shown that traces of agar react with the latter more readily than with guinea-pig serum.

The amount of agar which would be present in the dilutions A, B, C, D, and E, provided the original A mixture was not centrifugated, would correspond to 1 part of dry agar in 1000, 4000, 16,000, 64,000, 256,000 parts, respectively. The centrifugation of the mixture A obviously reduces the respective amounts of agar to a considerable extent. It has been pointed out heretofore that the sol-gel mixture 3, the agar-serum ratio of which is 1:40,000, will undoubtedly render guinea-pig serum toxic in dose of 3 c.c.; hence, even smaller amounts acting over a long time might be expected to have an action.

Ferment Adsorption.—In endeavoring to account for the action of the residual agar or trypanosomes, it might be assumed that such residues acted as adsorbing surfaces, and became laden with the ferment in much the same way that fibrin is supposed to take up thrombin. As a matter of fact, it has been shown by Hamburger⁸ that agar does adsorb digestive ferments. If something similar occurred in the course of anaphylatoxin-production, it should be possible to demonstrate the presence of an adsorbed enzyme, either by using such agar directly, or its extract, as a means of inducing toxicity.

A number of experiments were made with the object of detecting an adsorbed ferment. For this purpose, the precipitate obtained on centrifugation of the incubated agar-serum mixture was taken up in guinea-pig serum, previously inactivated at 56 C. for half an hour, and thoroughly mixed with the aid of a finely drawn-out pipet. Such mixtures were then incubated for varying lengths of time and tested. At the same time control mixtures of agar and distilled water were carried through exactly the same steps. Repeatedly, slight or fair shocks were obtained with the inactivated serum which had been treated with the supposedly ferment-laden agar, whereas the effect in the control tests was practically nil. The injection dose in these tests was 3 c.c. It seemed, therefore, as if the treated agar was able slightly to toxify the inactivated serum. The results, however, were by no means conclusive, and the slight effects could easily be ascribed to the more facile redistribution of the laden agar through the heated serum; in the controls the clean agar was not so easily dispersed.

These experiments were made on the assumption that the inactivation consisted in destroying the enzyme, the matrix being unaffected. In the light of subsequent work, it is quite likely that a change in the matrix does occur. If a laden agar such as was used in the experiments is added to normal guinea-pig serum, and the mixture tested at

⁸ Arch. néerl. d. sc. exact. et nat., 1908, 13, p. 428.

intervals of 15 minutes, it will be found to toxify, but the speed is not increased over that with untreated agar.

Another effort at demonstrating the presence of a ferment in the treated agar consisted in testing the action of aqueous extracts of the latter. The agar deposit obtained on centrifugation of an incubated sol-gel serum mixture was thoroughly rubbed up with distilled water, and this suspension was digested at 45 C. for 1 hour in the hope of dissolving out any adsorbed ferment. It was then centrifugated and the clear water extract was added to 1-1½ volumes of normal guinea-pig serum and incubated at 37 C. for 2, 3, and even 5 hours. The equivalent of 3 c.c. serum was injected at half-hour intervals. In 3 of these experiments, the results were practically nil; in a fourth, some dyspnea and moderate spasms were obtained in the tests made at 4 and 5 hours. This latter slight result was obtained with an extract of a deposit from a mixture which had been centrifugated at 8000 revolutions for 1 hour, in order to remove the agar as completely as possible; the extract was incubated with an equal volume of serum. The effects produced by the injection of 6 c.c. of this dilute serum are consequently those which could be expected from the incubation of diluted normal serum. All these attempts to prepare an active extract from the laden agar must be considered as negative.

Taken as a whole, the dilution experiments, as well as those with laden agar and with extracts of such, fail to give any clear or definite evidence of the participation of a ferment in anaphylatoxin-production in guinea-pig serum.

SUMMARY

The production of anaphylatoxin in guinea-pig serum through the aid of agar is a reaction of great speed. With proper conditions, incubation for from 8 to 10 minutes at 37 C. may render the serum fatal in dose of 3 c.c.

It is possible to reduce the lethal dose to 1 c.c.

The poison-production rapidly reaches a maximum, which appears to persist for a considerable time at 37 C.

The toxicity of such serum, when tested at regular intervals, appears to show more or less oscillation, as in the case of the trypanosome anaphylatoxin. Such variations are not due to a change in the amount of the poison, but to a varying resistance of the test animals.

The physical state of the agar is an important factor in the production of the poison. The 50 C. sol can be used to toxify serum, but the

0 C. gel apparently gives better results. The best results are obtained by the sol-gel method.

With the sol-gel method, a mixture (No. 3) representing 0.025 mg. of agar per cubic centimeter of serum, or 1:40,000, when incubated for an hour or more, became so toxic that 1.5 c.c. nearly proved fatal. This represents one-fortieth the amount of agar and one-third the dose employed by Bordet.

The most convenient mixtures are probably Nos. 4 and 5, with 0.025 and 0.05 c.c. of agar sol, respectively, per cubic centimeter of serum.

The reaction is not inhibited by an excess of agar, since a 1:1 mixture (No. 9) may render a serum toxic in dose of 1 c.c.

Some incubation of the agar-serum mixtures at about 37 C. is necessary. Mere contact at 0 C., even for many hours, does not result in poison-production—a fact which contraindicates adsorption as a factor in the reaction. Mixtures containing very small amounts of agar require a longer period of incubation than others.

The individual sera vary somewhat in the ease with which they can be toxified. This is not due to the presence of lipoidal matter, or to the age of guinea-pigs. The kind of food as in the case of rabbits may be a factor.

The poison persists in the treated serum after the complete removal of the agar by centrifugation at 8000 revolutions. It is not destroyed by heating such serum to 60 C. for half an hour.

No evidence was found to show that this anaphylatoxin was either preformed, or the result of ferment action. On the contrary, the findings oppose both views.

III. AGAR ANAPHYLATOXIN: RABBIT SERUM

F. G. NOVY AND P. H. DEKRUIF

SYNOPSIS

INTRODUCTION

INCONSTANCY OF RESULTS

METHODS

EXPERIMENTS WITH AGAR SOL (50 C.)

EXPERIMENTS WITH AGAR GEL (0 C.)

EXPERIMENTS WITH SOL-GEL AGAR

CENTRIFUGATION OF SOL-GEL MIXTURES

EFFECT OF ALKALI AND ACID ON TOXICITY

PRODUCTION OF ANAPHYLATOXIN AT 50, 55, AND 60 C.

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

RESISTANCE OF GUINEA-PIGS TO ANAPHYLATOXIN

SUMMARY

Bordet¹ stated that diverse fresh sera behave like that of the guinea-pig on contact with agar. It is to be presumed that this statement includes rabbit serum, but of that there is no certainty. Tchernor-outzky² specifically stated that the sera of rabbit, horse, and man, when treated with agar, become about as toxic as the serum of adult guinea-pigs (2 to 3 c.c. per 100 gm.). On the other hand, Haren³ reached the conclusion, based on insufficient data, that agar anaphylatoxin is toxic only for the homologous animal. In his tests he employed treated rabbit serum, which in dose of 2 to 3 c.c. produced no effect in guinea-pigs. It will be shown that his deductions were wrong.

There is no doubt but that rabbit serum, in general, is more difficult to toxify than is that of the guinea-pig. The least difficult in that respect is rat serum. The work with rabbit serum was included in this study for the reason that it is possible to obtain a large amount with the least outlay of time and expense. This fact to a large extent counterbalanced the difficulty or slowness in producing anaphylatoxin.

In a good portion of this work, the sera of fasting rabbits (2 to 24 hours) were employed in the belief that fasting gave a more uniform serum and made it more toxifiable. A brief fast for a few hours seemed to be better than one of a day or more. But even under these conditions, great variation in the ease or intensity of poison-production

¹ Compt. rend. Soc. de biol., 1913, 74, p. 227.

² Ibid., p. 1213.

³ Ztschr. f. Immunitätsf., 1914, 20, p. 676.

was encountered. A cloudy or lipoidal serum often gave better results than one which was water-clear. It is probable that the individual variation in the rabbits is a more important factor than it is with the guinea-pig or rat.

The uncertainty of results with rabbit serum proved often to be most disconcerting. Thus, an endeavor to repeat and extend the experiments given in Tables 35 and 42, made a year later, led to uniformly negative results. This striking difference could be accounted for in no other way than that the diet of the laboratory animals, on account of market conditions, was less varied than it had been before. The prohibitive cost of vegetables necessitated an almost exclusive hay diet, whereas in the previous year lettuce, cabbage, beets, carrots, bread, oats, etc., were freely given along with but little hay. A series of experiments, extending over more than a month, was made to locate the difficulty, but with no marked success. On a cabbage diet, the rabbits gave sera more promptly toxifiable with agar than on other food; but even this result was not dependable. A bread diet for a short time, likewise, appeared to favorably influence the serum. On the other hand, exclusive feeding of oats or carrots did not appear to make the serum more toxifiable. The most that can be said is that a daily varied diet is better than a single monotonous one.

No effort was made to reduce the lethal dose below 3 c.c., but it is quite possible that this can be done since the dose mentioned very often caused acute death in less than 3 minutes. The speed of poison-production, as in the case of guinea-pig and rat serum, is rapid, for frequently this dose proved to be fatal after an incubation of but 15 or 30 minutes. Failures, however, were more frequent, and with many rabbit sera, tho tests were made every 15 minutes, no fatal shock was obtainable until after 1½ to 2 hours, and sometimes even no result was obtained after incubation for twice that length of time.

The difficulty in toxifying a rabbit serum so that 3 c.c. represent more than 1 lethal dose accounts in part for the variable results in guinea-pigs. It will be shown at the end of this paper that the resistance of the guinea-pig is by no means uniform and this fact becomes more prominent the weaker the anaphylatoxin. Hence, the irregularity in results and the relatively few fatal shocks.

It must be borne in mind that normal untreated rabbit serum may produce acute death in dose of 3 c.c. This is especially true when it has been kept in contact with the spontaneous clot for some time, or when

bead-defibrination is resorted to. A full discussion of this question will be found in Part VIII, which deals with the primary toxicity of normal sera. It should be stated here that the sera for this work with agar were from perfectly normal rabbits and were always obtained by rod defibrination, a method which gives the least toxic serum; the acutely lethal dose of such sera is usually about 6 c.c.

When the physical condition of the rabbit is not taken into consideration, it is possible to obtain a serum which will have a greater initial toxicity. This appears to be the only explanation for some of the highly toxic rabbit sera obtained by Mita and Ito. Incidentally, it may be stated that we have found the serum of a cachectic rabbit to be acutely fatal in dose of 0.05 c.c. per 200 gm. of guinea-pig, a toxicity 120 times greater than that of the ordinary normal serum. It may be assumed that the matrix of the poison is increased in the sick animal, but it is equally reasonable to believe that this toxicity is due to a decrease in the amount of the antagonistic or calyptic substances present in the normal plasma.

It will be frequently found that a rabbit anaphylatoxin will produce a delayed death, one or more hours after injection. Such effects are to be ascribed to the primary toxicity of the serum, rather than to a fresh yield of anaphylatoxin. These facts indicate the need of parallel control tests with the same pooled serum, for without such careful controls an experiment can have but little value.

As regards the technic, it may be stated that the rabbits were always bled from the carotid, the blood being drawn directly into sterile carotid pipets, which have been described in Part I. The blood was immediately defibrinated with the glass rod, and then transferred to sterile special tubes and centrifugated at 8000 revolutions for from 5 to 30 minutes. The perfectly clear serum was usually employed at once, that is to say within about an hour after the start of bleeding. It can be toxified, however, even after it has been kept for 24 hours. The preparation of the agar, and its mode of employment were the same as given in Part II.

EXPERIMENTS WITH AGAR SOL, 50 C.

The first experiments with rabbit serum were made under conditions similar to those given in Table 19. The mixtures of 50 C. sol and serum, previously warmed to 37 C. (0.25:1), were thoroughly shaken for 1 minute, then centrifugated at 3000 revolutions for 5 minutes, either at once or after incubation at 37 C. for 2½, 5, 10, and 15

minutes. The centrifugated serum was then placed at 37 C. for 2 hours, after which it was tested. In one set of experiments, the equivalent of 3 c.c. of serum was injected; in another set an injection of 4 c.c. was used. The effects in both sets were slight or nil.

In another experiment, the sol-serum mixture being in the ratio of 0.025:1, and the incubation of the five portions before centrifugation being the same as given, of 5 tests with 4 c.c., 4 caused delayed deaths after some hours. Such protracted deaths show little or no increase over the primary toxicity of the serum employed. It will be seen, therefore, that the results by this method were less favorable than in the corresponding experiments with guinea-pig serum (Table 15).

EXPERIMENTS WITH AGAR GEL, 0 C.

The experimental conditions here corresponded with those for guinea-pig serum as given in Table 22. The iced gel was added to 4 parts of serum, previously cooled to 0 C.; the mixtures were thoroughly shaken for 2 minutes, after which they were placed at 37 C. for a variable time. They were then centrifugated at 3000 revolutions for 8 minutes; tests were made at once and after incubation for the time stated in Table 32. Three different sera were employed for these experiments; one was used for Expts. 1 and 2; another for Expts. 3, 4, and 5, and a third was used for Expts. 6 and 7. The protracted death in No. 4 must be ascribed to the primary toxicity of the serum used. The acute deaths in Nos. 9 and 11 are clearly due to anaphylatoxin.

TABLE 32

ACTION OF AGAR GEL (0 C.) ON RABBIT SERUM (0 C.) (RATIO 0.25:1. MIXTURE KEPT AT 37 C. THEN CENTRIFUGATED AND REINCUBATED)

Expt.	Guinea-Pig		Serum			Result
	No.	Weight	B. C. at 37 C.	A. C. at 37 C. (hr.)	c.c. (intravenously)	
I	1	175		3	4	Moderate shock
	2	170		19	"	Slight
II	3	178	10'	3	"	"
	4	171	10'	19	"	5½ hr.
III	5	202	30'		"	Very slight
	6	192	30'	4	"	Slight
IV	7	171	1 hr.		"	Very slight
	8	180	1 hr.	4	"	Slight
V	9	188	2 hr.		"	2:30"
	10	185	"	4	3	Slight
VI	11	171	"		4	4:50"
	12	175	"	7	"	Slight
VII	13	200	—	2	"	Slight

EXPERIMENTS WITH SOL-GEL AGAR

The sol-gel mixtures were prepared as described in connection with the work on guinea-pig serum. The mixtures were placed in cracked ice for 3 hours, after which a portion was removed and centrifugated at 3000 revolutions for 4 minutes and injected; the balance was placed at 37 C. and similarly tested every 15 or 30 minutes up to 3½ hours. The equivalent of 3 c.c. was injected in all tests. Only Mixtures 3 to 8 (Table 23) were made with rabbit serum. Mixtures 1 and 2 were not tried because of their failure to toxify guinea-pig serum.

One series of tests was made with each mixture in order to ascertain their relative toxifying values. The results of these tests will be briefly summarized. Mixtures 3 and 4 were made with one serum, 5 and 6 with another serum, and 7 and 8 with still another.

Mixture 3.—Fair shocks were obtained after incubation for ¼, 2, and 3½ hours; the test made at 3 hours killed in 3 minutes 20 seconds. It is evident that this mixture, having an agar-serum ratio of 1:40000, is capable of toxifying rabbit serum.

Mixture 4.—The test made after incubation for one-half hour killed in 8 minutes 20 seconds, while that at 3½ hours was likewise fatal in 3 minutes 30 seconds. A severe shock was obtained in the test made at 2 hours; the results of the other tests were slight.

Mixture 5.—The tests with this and the following mixture were made at intervals of 15 minutes up to 1¼ hours. The test made at one-half hour gave a good shock; those at 1 and 1¼ hours killed in 6 minutes and 3 minutes 10 seconds, respectively.

Mixture 6.—The immediate test gave a severe shock while that at one-quarter hour was moderate. The tests at ½, ¾, and 1 hour killed in 3 minutes, 3 minutes 35 seconds, and 3 minutes 45 seconds, respectively. This mixture, which represents an agar-serum ratio of 1:2000, was apparently the most active.

Mixture 7.—The tests made at once, and at 1½, and 2 hours gave fair shocks; those at ¾ and at 3½ hours killed in 13 and 4 minutes, respectively. The other tests gave but slight effects.

Mixture 8.—The agar-serum ratio in this mixture was 1:400, and this fact may have something to do with the protracted deaths. The serum used was of the same pool as that of Mixture 7, with which the deaths were acute or nearly so. In this set, the immediate test killed in 85 minutes, while that at one-half hour took 25 minutes. The other tests made up to 3½ hours had but slight effects.

A summary of these tests is given in Table 33. It will be seen that of 52 tests 12, or 23%, were fatal. The conclusion to be drawn is that rabbit serum can be toxified by the sol-gel treatment so that 3 c.c. will produce a typical acutely fatal shock. The optimal mixture appears to be No. 6. An excess of agar seems to be unfavorable.

TABLE 33

SUMMARY OF SOL-GEL RABBIT-SERUM MIXTURES, SHOWING DISTRIBUTION OF FATAL SHOCKS (MIXTURES KEPT AT 0 C. FOR 3 HR., THEN AT 37 C.; DOSE 3 C.C. SERUM)

Mixture	Ratio	Time When Tested in Hours											
		0	¼	½	¾	1	1¼	1½	2	2½	3	3½	
3	0.005:1	—	—	—	—	—	—	—	—	—	+	—	
4	0.025:1	—	—	+	—	—	—	—	—	—	—	+	
5	0.05 :1	—	—	—	—	+	+	—	—	—	—	—	
6	0.1 :1	—	—	+	+	+	—	—	—	—	—	—	
7	0.25 :1	—	—	—	+	—	—	—	—	—	—	+	
8	0.5 :1	+	—	+	—	—	—	—	—	—	—	—	

— = nonfatal effect
+ = death

The results shown in the preceding table must not be interpreted as constant or fixed for a given mixture. They will necessarily vary with the individual serum and in addition with the chance resistance of the guinea-pigs, as well as slight variations in the technic. Since Mixture 6 seemed to give the best results, it was used in nearly all the subsequent work and hence a number of these experiments are available for comparison. A summary of these experiments is given in Table 34, which shows only the fatal shocks. In all, the sol-gel mixtures were incubated till the end, only a small portion, the equivalent of 3 c.c. of serum, being removed, centrifugated, and tested at each period.

TABLE 34

SUMMARY OF EARLY TESTS WITH SOL-GEL RABBIT-SERUM MIXTURE 6, SHOWING DISTRIBUTION OF FATAL SHOCKS

Exper.	Time When Tested in Hours								
	0	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	$1\frac{1}{4}$	$1\frac{1}{2}$	$1\frac{3}{4}$	2
1	—	—	+	+	+	—			
2	—	—	—	—	—	—			
3	—	—	—	+	+	—			
4	+	—	—	—	—	—			
5	—	—	—	+	—	—	+	—	
6	—	—	+	—	—	—	+	—	
7	—	—	—	—	+	—	—	—	
8	+	—	+	—	—	—	—	—	+
9	—	—	—	—	—	—	+	—	—
10	—	+	—	—	—	—			
11	—	—	+						
12	—	+							
13	—	+							

— = nonfatal effect
+ = death

It will be seen that only 1 of the 13 experiments tabulated failed to produce a fatal shock. Similar experiments made a year later met with a considerably larger number of failures, the only apparent reason, as has already been pointed out, being a difference in diet. It is to be noted that the immediate tests made in Experiments 4 and 8 resulted in acutely fatal shocks; also that in 3 of the experiments the sera became fatally toxic after incubation for only 15 minutes. It will be seen further that of 76 tests 19, or 25%, proved fatal.

CENTRIFUGATION OF SOL-GEL MIXTURES

It has been shown that mixtures of agar and guinea-pig serum, when centrifugated at high speed immediately after icing, do not become toxic on subsequent incubation at 37 C. A similar experiment with rabbit serum gave likewise a negative result. Inasmuch as this experiment to a certain extent serves as a control for those which follow, it merits being given in a condensed form.

A sol-gel mixture, No. 6, consisting of 4.2 c.c. of sol and 42 c.c. of serum, both at 37 C., after vigorous shaking for 1 minute was placed in cracked ice for 2 hours. A small portion, representing 3 c.c. of serum, was then removed, centrifugated, and injected; the balance was centrifugated at 8000 revolutions for 1 hour, after which the clear treated serum was placed at 37 C. and tested at intervals of 15 minutes, up to 2 hours. The 10 consecutive tests made with this treated serum were uniformly negative, or at most had very slight effects.

This experiment, like that of the corresponding one with guinea-pig serum, shows that mere icing of the agar-serum mixture does not create a sufficient disturbance to toxify the serum. Were the reaction one of simple adsorption of an inhibiting substance, it would be reasonable to expect such adsorption to occur at a low temperature, to a moderate extent at least, and such treated serum after removal of the agar should give evidence of increased toxicity. Such, however, is not the case.

Incubation of the agar-serum mixture at about 37 C. is necessary to poison-production. After the reaction is once induced, the agar can be removed completely from the mixture and such treated serum on incubation will maintain its toxicity for some time, as will be seen from the following experiment.

A sol-gel mixture, No. 6, consisting of 6 c.c. of sol and 60 c.c. of serum was treated in the same way as the preceding, except that after

it had been iced 2 hours, and a portion removed for an immediate test (No. 1), the balance was placed at 37 C. for 15 minutes; then a portion was removed, centrifugated, and tested (No. 2) to see if the mixture had become toxic. This being the case, the entire mixture was immediately centrifugated at 8000 revolutions for 60 minutes; the resulting clear serum was tested at once, and the balance placed at 37 C. and tested at intervals of 15 minutes. The equivalent of 3 c.c. was used for all the injections.

A comparison of the results of this experiment, Table 35 and Chart 6, with those of a similar experiment with guinea-pig serum (Table 27) shows a marked agreement. In both, the toxicity persists after removal of the agar. The speed of poison-production is indicated by the fact that incubation for 15 minutes or less suffices to toxify. The apparent fluctuation in the toxicity must be ascribed to variations in the resistance of the recipients.

TABLE 35

CENTRIFUGATION AT 8000 R.P.M. APPLIED AFTER INCUBATION AT 37 C. FOR 15 MINUTES
(SOL-GEL MIXTURE 6, RATIO 0.1:1)

Guinea-Pig		Serum			Result
No.	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	c.c. (intra- venously)	
1	175	0		3	Very slight
2	170	15		"	3'15"
3	197		0	"	Moderate
4	171		$\frac{1}{4}$	"	3'
5	188		$\frac{1}{2}$	"	Slight
6	172		$\frac{3}{4}$	"	2'45"
7	205		1	"	Fair
8	176		$1\frac{1}{4}$	"	5'
9	179		$1\frac{1}{2}$	"	Slight
10	201		$1\frac{3}{4}$	"	Very slight
11	176		2	"	Severe
12	174		$2\frac{1}{4}$	"	3'
13	193		$2\frac{1}{2}$	"	12'
14	188		$2\frac{3}{4}$	"	Very slight
15	199		3	"	Slight

Inasmuch as it would be of interest to know how long this toxicity would persist in a treated rabbit serum one attempt was made to obtain such information. Unfortunately, the serum employed was not as reactive as that used for the preceding experiment. The sol-gel mixture, treated as described in the foregoing, on incubation for 15 minutes gave but very slight effect; tested again at the end of $\frac{1}{2}$, $\frac{3}{4}$, and 1 hour, it gave very severe shocks, but did not kill until after incubation for $1\frac{1}{2}$ hours. The mixture was then centrifugated at 8000 revolutions for 1 hour, and the clear serum thus obtained was placed at 37 C. No

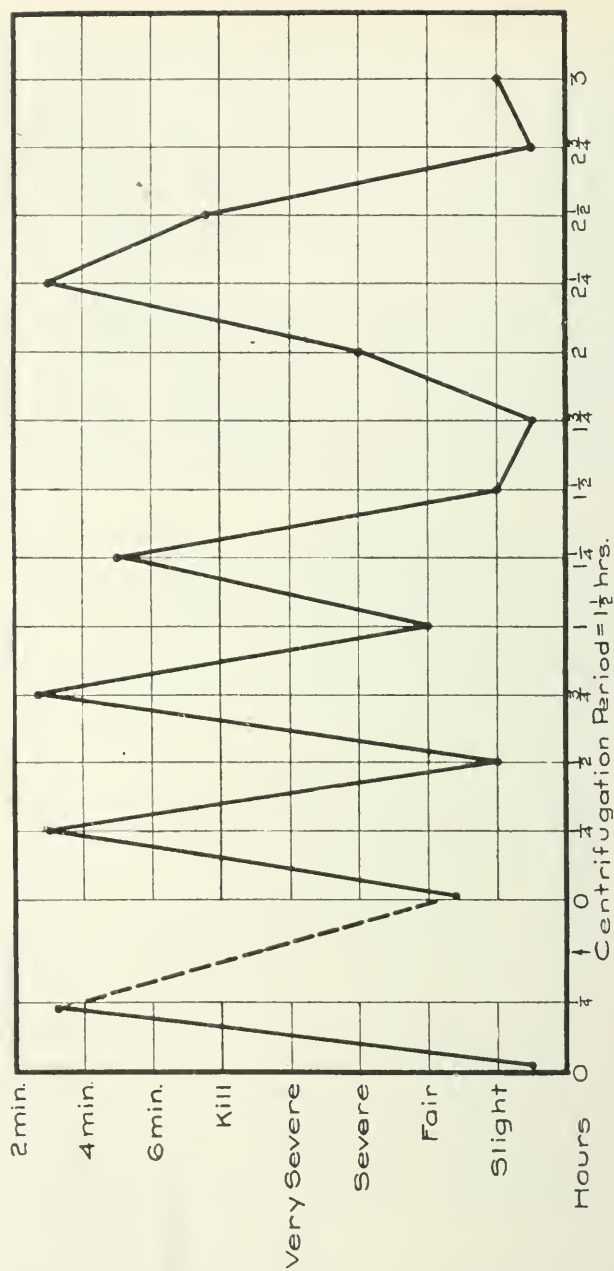


Chart 6. Centrifugation at 8000 r.p.m. of sol-gel rabbit-serum mixture No. 6, after incubation at 37 C. for 15 minutes (Table 35).

tests were made until the 12th hour; then it was tested at intervals of 15 minutes up to the 14th hour. The test at 12 $\frac{1}{4}$ hours killed in 3 minutes 50 seconds; of the other 7 tests only 1 was severe. The serum was allowed to remain at 37 C. and was again tested at quarter-hour periods, beginning at 24 hours. These tests gave but slight effects. In view of the fact that but 1 of 8 tests made at 12 hours was fatal, it is quite likely that a similar result might have been obtained after 24 hours.

This experiment, such as it is, shows that the toxicity may persist at 37 C. for 12 hours. With a serum of greater toxicity the result might have been more marked.

EFFECT OF ALKALI AND ACID ON TOXICITY

The only observations which have been made on the effect of alkali and acids on guinea-pig anaphylatoxin are those of Friedberger and Moreschi.⁴ They found that the addition of 0.1 c.c. of N NaOH to 3.5 c.c. of anaphylatoxin, the latter representing lethal doses of 1.5 c.c., did not destroy the poison after a contact of 6 minutes, whereas such contact for 2 hours at room temperature apparently did do so. The addition of 0.5 c.c. of N NaOH seemed to destroy it in 6 minutes as well as in 2 hours. The animals which survived the injection of the alkalinized serum died, however, within 24 hours. On the other hand, the addition of corresponding amounts of N HCl and contact for 2 hours apparently had no effect on the toxicity. The additions mentioned correspond to 0.0286 and 0.143 c.c. normal solution per cubic centimeter of serum.

In view of the importance of these results it was desirable to make a more extended study of the effects of acid and alkali not only upon the poison, but also upon the production of anaphylatoxin. This work was carried out with rabbit serum, tho it would have been better to have made similar experiments with rat serum.

Before beginning this work the alkalinity of several normal rabbit sera was approximately determined by titration with N/20 HCl, using a sensitive litmus paper as an indicator. For this purpose 5 c.c. of the serum were placed in a large evaporating dish, distilled water added, and the whole warmed to 40 C. before adding the acid. The neutral point was apparently reached by the addition of 0.024 c.c. normal acid per cubic centimeter of rabbit serum. The addition of 0.03 c.c. gave a distinct acidity. The sera of fasting rabbits apparently required a trifle more acid, namely, 0.0265 to 0.027 c.c.; this alkalinity was not increased by heating such serum at 60 C. for $\frac{1}{2}$ to 1 hour, but the heating seemed to make

⁴ Berl. klin. Wchnschr., 1912, 49, p. 743.

the protein more coagulable by the acid. Tested in the same way, a guinea-pig serum required 0.023 c.c., while a rat serum took 0.027 c.c. normal acid per cubic centimeter of serum. It would seem therefore that the slight differences in the alkalinity of these three kinds of sera would hardly account for their variability as regards anaphylatoxin-production. It may be added that, after treatment with agar, the rabbit serum appears to show a lessened alkalinity (0.019 to 0.021 c.c.).

For the following experiments, sol-gel mixtures (No. 6) were prepared in the usual way, then placed in cracked ice for 2 hours. A small portion was then removed, centrifugated, and tested at once; the balance was placed at 37 C., and like portions were removed and similarly treated at intervals of 15 minutes. In all tests, the equivalent of 3 c.c. of serum was injected.

For each experiment a single rabbit serum, previously warmed to 37 C., was divided into 2 portions of 27 c.c. each. To one of these was added the desired amount of normal Na_2CO_3 or HCl , and to the other portion, serving as a control, a corresponding amount of distilled water. The two portions were then treated with the 37 C. sol, and eventually tested as indicated. It will be noted, therefore, that for each experiment there is a corresponding control. The two portions were incubated at the same time, each control being injected about a minute after the regular test animal. It would have been preferable if one pooled serum had been used for all three of the following experiments, but this, at the time, seemed impracticable.

For the first experiment (Table 36), the normal alkalinity was tripled by the addition of 0.048 c.c. normal Na_2CO_3 per cubic centimeter of serum. It will be noted that there is no evidence of poison-production in such alkaline serum, tho the duration of the experiment was 2 hours; the corresponding control series gave 2 fatal shocks. An excess of alkali appears to inhibit the generation of the anaphylatoxin. This is in accord; as it were, with the findings of Friedberger and Moreschi that alkali destroys the ready-made anaphylatoxin. This experiment is deserving of repetition with the more reactive rat serum.

TABLE 36
PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM OF TRIPLE ALKALINITY (SOL-GEL MIXTURE 6. RATIO 0.1:1. DOSE 3 C.C.)

0.024 c.c. N Na_2CO_3 per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	208	0	Nil	1	199	0	4'20"
2	196	$\frac{1}{4}$	Slight	2	209	$\frac{1}{4}$	Nil
3	210	$\frac{1}{2}$	Very slight	3	205	$\frac{1}{2}$	3'
4	176	$\frac{3}{4}$	" "	4	198	$\frac{3}{4}$	Nil
5	181	1	Slight	5	183	1	Slight
6	211	$1\frac{1}{4}$	Very slight	6	209	$1\frac{1}{4}$	Very slight
7	207	$1\frac{1}{2}$	Slight	7	195	$1\frac{1}{2}$	Nil
8	199	$1\frac{3}{4}$	Very slight	8	188	$1\frac{3}{4}$	"
9	177	2	" "	9	187	2	Slight

In the second experiment (Table 37), the alkalinity was doubled by the addition of 0.024 c.c. N Na_2CO_3 per cubic centimeter of serum,

with the surprising result that the toxicity was decidedly increased as compared with that of the corresponding control. Unless this is an error incidental to the variable resistance of guinea-pigs, it would indicate that a moderate increase in the alkalinity favors the production of anaphylatoxin. It should be noted, however, that Friedberger and Moreschi employing a trifle more alkali (0.0286 c.c. N NaOH), as compared with the foregoing amount, destroyed the ready-made anaphylatoxin after contact for 2 hours at room temperature.

TABLE 37

PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM OF DOUBLE ALKALINITY (SOL-GEL MIXTURE 6. RATIO 0.1:1. DOSE 3 c.c.)

0.024 c.c. N Na ₂ CO ₃ per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	204	0	Slight	1	187	0	Very slight
2	170	1/4	Very slight	2	175	1/4	Very slight
3	206	1/2	9'45"	3	205	1/2	Nil
4	190	3/4	5'50"	4	209	3/4	Very slight
5	171	1	Very slight	5	176	1	3'50"
6	183	1 1/4	Very slight	6	208	1 1/4	Very slight
7	183	1 1/2	Near-kill	7	205	1 1/2	Slight
8	174	1 3/4	Nil	8	195	1 3/4	Very slight
9	207	2	3'25"	9	173	2	Slight

In the third experiment (Table 38) the serum was rendered sesqui-alkaline by the addition of 0.012 c.c. N Na₂CO₃ per cubic centimeter of serum. Here the increased anaphylatoxin-production was even more striking than in the preceding since every test made during the 2 hours' experiment gave either a very severe shock or a kill. The control tests, while not so severe, showed that the serum used was more reactive than those employed in the two preceding experiments. Further tests of sesqui-alkaline serum are desirable in view of these results.

TABLE 38

PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM OF SESQUI-ALKALINITY (SOL-GEL MIXTURE 6. RATIO 0.1:1. DOSE 3 c.c.)

0.012 c.c. N Na ₂ CO ₃ per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	195	0	3'5"	1	201	0	Nil
2	195	1/4	Very severe	2	175	1/4	Near-kill
3	171	1/2	Very severe	3	170	1/2	4'
4	180	3/4	Very severe	4	202	3/4	Moderate
5	199	1	3'45"	5	203	1	Nil
6	206	1 1/4	3'10"	6	210	1 1/4	Nil
7	205	1 1/2	Very severe	7	209	1 1/2	5'
8	182	1 3/4	3'5"	8	170	1 3/4	Fair
9	175	2	Near-kill	9	171	2	3'10"

In the fourth experiment (Table 39) the serum was rendered semi-neutral by the addition of 0.012 c.c. N HCl per cubic centimeter of serum. In this set there is a marked agreement with the control, showing that a slight decrease in the alkalinity is without effect on the production of anaphylatoxin.

TABLE 39
PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM, SEMINEUTRAL (SOL-GEL MIXTURE 6.
RATIO 0.1:1. DOSE 3 C.C.)

0.012 c.c. N HCl per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	193	0	Slight	1	187	0	Slight
2	198	$\frac{1}{4}$	Very slight	2	182	$\frac{1}{4}$	Very slight
3	171	$\frac{1}{2}$	Moderate	3	173	$\frac{1}{2}$	Very slight
4	186	$\frac{3}{4}$	3'40"	4	181	$\frac{3}{4}$	2'15"
5	201	1	Nil	5	170	1	Severe
6	187	1 $\frac{1}{4}$	Severe	6	172	1 $\frac{1}{4}$	Moderate
7	179	1 $\frac{1}{2}$	4'	7	184	1 $\frac{1}{2}$	3'15"
8	170	1 $\frac{3}{4}$	Very severe	8	172	1 $\frac{3}{4}$	Moderate

In the fifth experiment (Table 40) the serum was very faintly acidified by the addition of 0.03 c.c. of N HCl per cubic centimeter of serum. This series of tests was discontinued at the end of 1 $\frac{1}{4}$ hours, and up to the end of that time the results indicated a decrease, if not suppression, of anaphylatoxin-production. The experiment should be repeated and extended, especially since the control showed but a moderate reactivity; the only fatal result, that in the immediate test made after icing (No. 1), may well be due to the use of a very susceptible guinea-pig. In view of the results of Friedberger and Moreschi, it might be expected that acid would have no influence on the poison-production.

TABLE 40
PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM, FAINTLY ACIDIFIED (SOL-GEL MIXTURE 6.
RATIO 0.1:1. DOSE 3 C.C.)

0.03 c.c. N HCl per c.c. Serum				Control with Same Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	172	0	Slight	1	181	0	3'45"
2	205	$\frac{1}{4}$	Very slight	2	207	$\frac{1}{4}$	Slight
3	176	$\frac{1}{2}$	Slight	3	202	$\frac{1}{2}$	Moderate
4	185	$\frac{3}{4}$	Very slight	4	172	$\frac{3}{4}$	Fair
5	195	1	Very slight	5	171	1	Slight
6	206	1 $\frac{1}{4}$	Moderate	6	200	1 $\frac{1}{4}$	Very slight

In a sixth experiment (Table 41) the conditions were changed; the object being to ascertain the effect produced by an excess of acid and alkali when allowed to react with sera for 2 hours at 37 C. The serum

was divided into 2 portions; one received 0.048 c.c. N HCl per cubic centimeter, while the other received 0.048 c.c. N Na₂CO₃ per cubic centimeter. After these mixtures had been incubated for 2 hours at 37 C., they were adjusted to approximately the original reaction by the addition of 0.048 c.c. of normal alkali to the former, and the same amount of normal acid to the latter. They were then treated by the sol-gel method in exactly the same way as in the preceding experiments. It is significant that on incubation these sol-gel mixtures did not give the usual precipitate, behaving in this respect like inactivated serum (Bordet).

The results in this experiment were striking, tho unfortunately no parallel control tests were made with the same serum untreated with chemicals. As seen in the table, the acidified serum showed practically no evidence of poison-production up to 2 hours, the duration of the experiment. The alkalinized serum showed the same behavior, but the last, or 2-hour, test did cause acute death. Whether this means that the poison was beginning to form at that time, or that by chance a very susceptible guinea-pig was then used, cannot be determined without further tests.

It would appear from this experiment that a serum is denatured by previous treatment with acid or alkali, the former perhaps being more active than the latter. The results of the acid treatment seem to confirm those of the preceding experiment. Similarly, the effects with alkali-treated serum correspond with those obtained in the first experiment (Table 36), where the same amount of alkali was used in the presence of the sol-gel.

TABLE 41

PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM TREATED WITH ACID (OR ALKALI) FOR 2 HOURS AT 37 C. BEFORE ADDITION OF AGAR (SOL-GEL MIXTURE 6. RATIO 0.1:1. DOSE 3 c.c.)

0.048 c.c. N HCl per c.c. Serum				0.048 c.c. N Na ₂ CO ₃ per c.c. Serum			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 37 C.	Result
1	210	0	Very slight	1	205	0	Very slight
2	208	1/4	Very slight	2	202	1/4	" "
3	198	1/2	Nil	3	203	1/2	" "
4	186	3/4	Nil	4	183	3/4	" "
5	180	1	Very slight	5	175	1	" "
6	205	1 1/4	Slight	6	170	1 1/4	" "
7	207	1 1/2	Slight	7	172	1 1/2	Nil
8	209	1 3/4	Very slight	8	183	1 3/4	Very slight
9	211	2	Very slight	9	177	2	2'50"

The general conclusions to be drawn from these experiments are: first, that an excess of alkali (0.048 c.c. per cubic centimeter) alters

the serum and prevents or greatly retards the anaphylatoxin-production; second, a moderate increase in alkalinity may actually favor its production; third, a slight decrease in alkalinity is without appreciable effect; and fourth, a faint acidity may retard, while an excess of acid (0.048 c.c. per cubic centimeter) may alter the serum so that poison-production is suppressed. An excess of acid as well as of alkali therefore interferes with the production of anaphylatoxin.

Action of Acid on Anaphylatoxin.—The effects observed when an excess of acid or of alkali was added to the agar-serum mixtures might be interpreted as due to a weakening or destruction of the hypothetical ferment. It is more probable, however, that the very labile matrix is involved in the reaction. The poison once formed may be looked upon as relatively stabile, a fact which is indicated by its behavior under the application of heat at 60 C. or 70 C. Since it had been shown that the toxicity of a treated serum persists after the removal of the agar (Table 35), it seemed desirable to test the action of acid and of alkali on such anaphylatoxin repeating, as it were, the work of Friedberger and Moreschi with guinea-pig anaphylatoxin.

For the following experiment (Table 42) a serum (68 c.c.) was treated by the sol-gel method, the procedure being exactly the same as that used for the experiments of Table 35. After the first fatal shock had been obtained (No. 3), the mixture was centrifugated at 8000 revolutions for 1 hour. The clear serum when tested at once was fatal (No. 4). It was then placed at 37 C. and after the next fatal shock (No. 6) it was divided into 2 portions: to one portion, 0.072 c.c. N HCl per cubic centimeter of serum was added, while the other portion served as a control. Both were then returned to the water-bath at 37 C. and tested at intervals of 15 minutes. The equivalent of 3 c.c. serum was used as the dose, and before injection this was neutralized by the addition of 0.3 c.c. N Na_2CO_3 . The tests of control and corresponding treated serum were made together at each test period.

The results are given in Table 42 and shown also in Chart 7. It will be observed that the control tests (Nos. 7 to 12) show persistence of the poison, tho one-half of the animals show very little effect, because of individual resistance. The tests with acid-treated serum (Nos. 7a to 14a) seem to show a marked drop in toxicity, since but 1 of 8 proved fatal and the others were all weak. It would appear reasonable to assume from this experiment that some of the poison had been destroyed by the acid, but this cannot be considered as established without further trials.

TABLE 42
 BEHAVIOR OF ANAPHYLATOXIN TOWARD N HCL (0.072 C.C. PER C.C. SERUM)

Control: Portion Untreated with Acid					Portion Treated with Acid			
Guinea-Pig	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	Result	Guinea-Pig	Weight	With Acid at 37 C. (hr.)	Result
1	187	0		Very slight	← Mixture centrifugated at 8000 r.p.m. for 60'			
2	175	15		Nil				
3	189	30		3'40"				
4	181		0	4'50"	← Mixture divided, one-half treated with acid			
5	170		1 $\frac{1}{4}$	Slight				
6	198		1 $\frac{1}{2}$	3'50"				
7	200		3 $\frac{3}{4}$	Very slight	7a	188	1 $\frac{1}{4}$	Moderate
8	175		1	4'5"	8a	182	1 $\frac{1}{2}$	Slight
9	205		1 $\frac{1}{4}$	Very slight	9a	201	3 $\frac{3}{4}$	Very slight
10	189		1 $\frac{1}{2}$	4'	10a	175	1	Nil
11	182		1 $\frac{3}{4}$	5'10"	11a	170	1 $\frac{1}{4}$	Very slight
12	198		2	Nil	12a	201	1 $\frac{1}{2}$	3'55"
13			2 $\frac{1}{4}$		13a	171	1 $\frac{3}{4}$	Slight
14			2 $\frac{1}{2}$		14a	207	2	Very slight

The foregoing experiment was repeated with this difference, that as soon as the sol-gel mixture 6 became toxic, which occurred after incubation for 15 minutes, it was divided into 2 portions: to one portion, 0.072 c.c. N HCl per cubic centimeter of serum was added; the other portion was retained as a control. Both portions were then centrifugated at 8000 revolutions for 15 minutes, and then tested at once, the balance of each portion being placed at 37 C. and retested at intervals of 15 minutes. As before, the equivalent of 3 c.c. of serum was used as the test dose. In the case of the acid-treated serum, this was neutralized by the addition of 0.3 c.c. N Na₂CO₃ before injection.

 TABLE 43
 BEHAVIOR OF ANAPHYLATOXIN TO N HCL (0.072 C.C. PER C.C. SERUM)

Control: Portion Untreated with Acid					Portion Treated with Acid			
Guinea-Pig	Weight	B. C. at 37 C. (min.)	A. C. at 37 C. (hr.)	Result	Guinea-Pig	Weight	With Acid at 37 C. (hr.)	Result
1	210	0		Moderate	← Mixture divided; acid added and both centrifugated at 8000 r.p.m. for 15'			
2	176	15		27'				
3	180		0	Slight				
4	170		1 $\frac{1}{4}$	3'30"	3a	185	0	3'40"
5	210		1 $\frac{1}{2}$	3'20"	4a	176	1 $\frac{1}{4}$	Near-kill
6	207		3 $\frac{3}{4}$	Very slight	5a	195	1 $\frac{1}{2}$	Slight
7	196		1	Very slight	6a	180	3 $\frac{3}{4}$	Slight
8	174		1 $\frac{1}{4}$	Very severe	7a	206	1	Slight
9	172		1 $\frac{1}{2}$	Slight	8a	203	1 $\frac{1}{4}$	Near-kill
10	185		1 $\frac{3}{4}$	Very slight	9a	202	1 $\frac{1}{2}$	4'20"
11			2		10a	171	1 $\frac{3}{4}$	4' 5"
					11a	209	2	Moderate



Chart 7. Behavior of anaphylatoxin to N HCl; the lighter line is the acid curve, the heavy one is that of the untreated portion (Table 42).

The results of these tests are shown in Table 43. It will be seen that the toxicity of the acid-treated serum (3a to 11a) is fully as great, if not more so, than that of the control portion (Nos. 3 to 10). While it is probable that some adsorption of the acid by the agar took place, it is unlikely that this occurred to any great extent in view of the short contact before centrifugation. It must be concluded from this experiment that the anaphylatoxin is not affected by the acid in the concentration given.

PRODUCTION OF ANAPHYLATOXIN AT 50 C.

Since the agar-serum mixture does not become toxic when kept in ice, but does become so when incubated at 37 C., it seemed as if at a still higher temperature the poison-production might be more marked and also more rapid. This certainly could be expected if dispersion was the sole factor in the reaction, provided, however, that such increased temperature did not induce changes in the dispersed colloid.

The first experiment made with this object in view ended in a wholly negative result. A sol-gel mixture (No. 6) was prepared in the usual way, and after being kept at 0 C. for 2 hours was placed in a Roux water bath at 50 C. Nine consecutive tests, made at intervals of 15 minutes, up to 2 hours, with the equivalent of 3 c.c. of serum as the test dose, had practically no effect. The conclusion seemed justified that at this temperature, the poison-production could not take place. However, since this experiment was not accompanied by a parallel control, with a portion of the same serum kept at 37 C., the result was open to doubt. Accordingly, the experiment was repeated, provision this time being made for a rigid control.

The sol-gel mixture was prepared as before, and placed for 2 hours at 0 C. A portion, the equivalent of 3 c.c. of serum, was then removed, centrifugated, and tested (No. 1); the remainder was in turn divided into 2 portions, one of which was placed at 37 C., and the other at 50 C. At intervals of 15 minutes, portions were removed from each of these and tested as before. The parallel tests were made within 2 or 3 minutes of each other. As will be seen from Table 44, the two portions (Tests 2 to 4 and 2a to 5a) behaved exactly alike. After Test 5a was made, the mixture kept at 50 C. was in turn divided into 2 parts; one remained at 50 C., and the other at 37 C. Both portions were tested, as before, at intervals of 15 minutes; they again showed a marked agreement in results.

Apparently, anaphylatoxin can be produced by the incubation of a sol-gel mixture at 50 C., and about as well as at 37 C. This result corresponds to the production of toxicity in normal rabbit blood which is allowed to clot at 50-60 C.

There can be no doubt but that the portion which was placed at 50 C. developed anaphylatoxin, but it may be questioned whether this poison was produced at that temperature. The iced agar-serum mixture when placed in the water bath, necessarily warmed up slowly, and it is more than probable that the poison was produced before the mixture was heated up to 50 C. The control test 2 shows that the poison was produced at 37 C. within 15 minutes; to raise the temperature of the iced mixture to 50 C. may require from 5 to 8 minutes, and this time is sufficient to make the poison, especially as the optimal temperature is probably about 45 C.

In view of these considerations it is not surprising to find that iced agar-serum mixtures when placed at 55 C., or even at 60 C., likewise develop anaphylatoxin. Here also it is to be understood that the poison is produced en route, as it were, before the temperature reaches or passes 50 C.

When a rabbit serum is dialyzed in a collodium sac against distilled water at 50 C. for a quarter to a half hour, and is then treated by the sol-gel method and the mixture placed at 37 C., it will be found that very little poison is produced. It follows that even a short exposure of the serum at 50 C. causes some change or modification in the matrix of the poison. This effect is more marked when the dialysis takes place at 55 C. The results are most conclusive when the more reactive rat serum is employed for such tests. Experiments with this serum will be considered in Part IV.

TABLE 44
PRODUCTION OF ANAPHYLATOXIN IN RABBIT SERUM AT 50 C.

Portion at 37 C.				Portion at 50 C.			
Guinea-Pig	Weight	Hours at 37 C.	Result	Guinea-Pig	Weight	Hours at 50 C.	Result
1	170	0	Very slight	2a	172	1/4	3'20"
2	191	1/4	5'40"	3a	205	1/2	Moderate
3	171	1/2	Fair	4a	211	3/4	Slight
4	188	3/4	Very slight	5a	186	1	Very slight
5							
6*	181	1/4	5'25"	6a	176	1 1/4	2'45"
7	207	1/2	Very slight	7a	185	1 1/2	Slight
8	187	3/4	Severe	8a	193	1 3/4	Very slight
9	185	1	Near-kill	9a	197	2	Very slight
10	180	1 1/4	Slight	10a	173	2 1/4	Slight

* After Test 5a the 50 C. mixture was divided and one half placed at 37 C. (Nos. 6 to 10).

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

Many attempts were made, similar to those employed in connection with the study of guinea-pig serum, to detect the presence of a ferment. The serial dilutions A and B of treated centrifugated sera with normal rabbit serum were prepared, and incubated at 37 C., after which they were tested at diverse periods (2 to 24 hours). Usually, little or no effect was obtained, but, at times, the portions tested at the 18- or 24-hour period caused death in the course of several hours. The test dose was 4 c.c., and this fact together with that of the prolonged incubation eventually led to the realization that such deaths were really due to the inherent primary toxicity of the serum.

It was hoped that better results would be obtained by diluting the treated centrifugated rabbit serum with normal rat serum. The fact that the latter is very readily toxifiable seemed to make it a reagent of choice for the detection of the ferment. When the dilutions A, B, and C were prepared with rat serum and incubated at 37 C. for from 2 to 5 hours and then tested, it was found that in dose of 1 c.c. they did produce acutely fatal shocks. Such results might be taken to indicate ferment action were it not for the fact that control tests demonstrated that they were due to the toxifying action of the residual agar, the trace left in the treated rabbit serum after centrifugation at 3000 revolutions. The presence of such residual agar was demonstrable, as in the case of guinea-pig serum, by recentrifugation at high speed.

Were a ferment concerned in the production of anaphylatoxin, it would be reasonable to expect that the toxicity would progressively increase in the agar-treated serum. It will be seen, however, by a study of the tables that the initial more or less rapid production of poison is not followed by an appreciable increase. Such being the case, it is hardly possible for the dilutions to give other results than those obtained. This method of approach has therefore failed to give any evidence of the participation of an enzyme in the production of anaphylatoxin.

RESISTANCE OF GUINEA-PIGS TO RABBIT ANAPHYLATOXIN

The apparent oscillation in the toxic effects, as noted in guinea-pigs after the injection of anaphylatoxin of diverse origin (including that of primarily toxic sera), was interpreted for a time as indicating a periodicity or wave formation of the poison. This view implying a variability in the poison and a uniformity in the resistance of the

guinea-pig was definitely abandoned as the result of an extended series of tests made with rabbit anaphylatoxin. It was found that the resistance of the guinea-pig to the poison varies considerably, and that the production of little or no effect in an animal does not necessarily imply the absence of anaphylatoxin. This fact became apparent when, in making serial tests at one-quarter hour intervals, 2 guinea-pigs of the same weight were injected each time with the same material; the two injections being made within half a minute of each other. In all the tests referred to in the following, the agar rabbit anaphylatoxin was used in dose of 3 c.c.

An analysis of 110 pairs of such tests, showed that in 55 pairs (50%) the effects were exactly alike, the two individuals of each paired test responding in the same manner; that is, 2 acute deaths, or 2 severe shocks, or 2 nil effects.

In 27 pairs the reaction was nearly alike, the 2 animals of each double test showing some difference, but not marked. Thus, of 2 guinea-pigs receiving the same dose at the same time, one might die of acute shock and the other might experience a very severe shock or near-kill. Such variation in individuality was to be expected.

On the other hand, a third group of 28 pairs of tests (25%) showed, rather unexpectedly, an extreme variation. This group includes those paired tests in which one animal dies, or receives a very severe shock, while its mate shows little or no effect. The results obtained in 8 pairs of such tests taken at random, are given in Table 45. For other inoculations in pairs, see Tables 55 and 56.

TABLE 45
SHOWING EXTREME INDIVIDUAL RESISTANCE TO SIMULTANEOUS INJECTION OF THE SAME
DOSE OF ANAPHYLATOXIN IN PAIRED GUINEA-PIGS

Guinea-Pig		Result
No.	Weight	
1	200	3'25"
1a	201	Very slight
2	187	Slight
2a	186	0'5"
3	205	4'40"
3a	204	Slight
4	190	2'40"
4a	188	Nil
5	194	Slight
5a	196	3'50"
6	198	2' 5"
6a	198	Very slight
7	195	Slight
7a	197	3'17"
8	185	Slight
8a	185	2'35"

In view of these results it is evident that one cannot speak of a strictly minimal lethal dose. The amount of poison necessary to produce a fatal shock in every one of a series of 8 or 10 animals is probably at least twice that which gives an occasional kill. Furthermore, great care must be taken in drawing conclusions from serial tests in which but 1 or 2 die of 8 or 10 animals. A death or severe shock does not necessarily mean an increase in the amount of the poison, and per contra the absence of a severe shock does not mean that the poison is not present. And again, it is obviously wrong to assume (as has often been done in the past) that the poison has been destroyed when a single test (or even a number of single tests) happens to be negative. In such cases, a multiple dose and duplicate tests are essential to avoid error.

SUMMARY

The production of anaphylatoxin in normal rabbit serum is not as facile as in guinea-pig or rat serum.

The individual sera show considerable difference in the ease with which they yield the poison. The presence of lipoidal matter is not inhibitive. The sera after a short fast appear to be more reactive. A varied diet appears to be favorable to the reaction.

The primary toxicity of the normal serum is greatly influenced by the method of defibrination as well as by the state of the animal. The sera obtained by rod defibrination may be toxic in dose of 6 c.c. The sera from cachectic treated rabbits may cause acute fatal shock in dose of 0.05 c.c., which represents a toxicity 120 times greater than that of normal serum.

Normal rabbit serum can be toxified with agar so that 3 c.c. will be fatal.

Attempts at toxifying with agar sol (50 C.), or gel (0 C.), were less favorable than like experiments with guinea-pig serum.

The sol-gel method appears to be the best means of inducing poison-production. Mixture 3, which contains but 0.025 mg. of agar per cubic centimeter of serum (1:40000), can become fatally toxic. The optimal mixture is probably No. 6. An excess of agar appears to have an unfavorable action.

The poison is not produced at 0 C.; incubation at about 37 C. is necessary. The apparent production of anaphylatoxin in agar-serum mixtures incubated at 50 C. or higher really occurs before such high temperature is reached.

The speed of poison-production is considerable, suitable sera being toxified in less than 15 minutes. The poison once developed persists in the serum even after complete removal of the agar by high-speed centrifugation. In such serum, the toxicity may persist at 37 C. for more than 12 hours.

An excess of acid as well as of alkali interferes with the production of anaphylatoxin. A moderate increase in the alkalinity may increase the anaphylatoxin-production. The ready-made anaphylatoxin is not affected by a moderate acidity.

Dialysis of rabbit serum in collodium sacs at 50 C. renders it less toxifiable. This fact as well as the behavior with excess of acid or alkali indicates that the matrix of the poison is labile.

Attempts to demonstrate the participation of a ferment in the reaction failed.

Marked variations in the susceptibility of guinea-pigs to the rabbit anaphylatoxin are possible. This fact explains the apparent oscillation in the toxicity of normal as well as of treated sera.

IV. AGAR ANAPHYLATOXIN: RAT SERUM

F. G. NOVY AND P. H. DEKRUIF

SYNOPSIS

INTRODUCTION

INCONSTANCY OF RESULTS

METHODS

EXPERIMENTS WITH AGAR SOL (37 C.)

EXPERIMENTS WITH AGAR GEL

EXPERIMENTS WITH SOL-GEL AGAR

APPARENT VARIATIONS IN TOXICITY

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

SERIAL DILUTIONS. EFFECT OF CENTRIFUGATION

HEATING OF MIXTURE TO 60 C. BEFORE INCUBATION

ACTION OF TREATED SERUM ON HEATED NORMAL SERUM

ACTION OF AGAR DEPOSIT ON HEATED NORMAL SERUM

ATTEMPTS TO EXTRACT FERMENT FROM AGAR DEPOSIT

ACTION OF HEATED TREATED SERUM ON NORMAL SERUM

ACTION OF AGAR ON HEATED NORMAL SERUM

TOXICITY OF THE AGAR DEPOSIT

SUMMARY

The early work on trypanosomes demonstrated that rat serum gives rise to a very active anaphylatoxin, and consequently, in the major part of the agar studies this reagent was used. There is no evidence to show that rat serum has been used for this purpose by other investigators and yet, as will be shown, it is incomparably the best,* since it can be toxified in a few minutes to such an extent that 0.25 c.c. will cause typical acute fatal shock. The speed and ease of the poison-production are remarkable when it is borne in mind that the primary toxicity of normal rat serum is no greater than that of the guinea-pig or rabbit.

In the beginning, considerable variation in results was encountered, just as in the work with guinea-pig and rabbit serum. It was reasonably clear, however, that this diversity was chiefly due to imperfect conditions as regards distribution of the agar in the serum, and for that reason much time and endeavor were devoted to developing the best methods of work. But even with improved methods it was found that occasional variation was encountered, which served to show that the rat serum was not always uniform; a fact which corresponds with the observations made in regard to the sera of the guinea-pig and rabbit.

The rat blood was drawn from the heart and defibrinated in the tube pipet by means of the glass rod, the technic being the same as that given in Part I. As a rule, pooled fresh sera were employed, but equally good results were

* The serum of the musk-rat (*Castor zibethicus*) is probably as toxifiable as that of the rat. In one experiment with sol-gel Mixture 6, after incubation at 37 C. for 30 minutes, the serum in dose of 0.5 c.c. gave a typical fatal shock in 4 minutes 45 seconds in a guinea-pig weighing 173 gm.; 0.25 c.c. gave a severe shock.

obtained with sera which had been iced or kept at room temperature for 24 hours. The presence of lipoids did not seem to interfere with the poison-production. Serum prepared in this way is ordinarily not harmful, in dose of from 5 to 6 c.c. given intravenously, to 200-gm. guinea-pigs. But, after incubation at 37 C. for some minutes, it may produce an acutely fatal anaphylactic shock in dose of 4 c.c. (Part VIII). It has even at times been found to produce the same effect in dose of 1 c.c., when the incubation had extended over a long period of time (20 to 120 hours). In this autotoxification, the anaphylatoxin is produced without the known intervention of any foreign agent. This fact indicates an inherent lability in the serum which is merely accentuated or accelerated by the presence of alien substances, such as agar, trypanosomes, distilled water, etc.

The preparation of the agar solution has already been described. As pointed out, it can be sterilized at 100, 110, 120, 130, or 140 C. without affecting the toxifying power. Further evidence of this will be presented in Table 47.

EXPERIMENTS WITH AGAR SOL, 37 C.

At the outset, solid or semisolid gel was used, but frequent irregularities and failures seemed to indicate that the trouble was due largely to inability to duplicate a given state of division. Accordingly, it was hoped to overcome this difficulty by the use of a hydrosol. For this purpose, the agar after being liquefied in the water bath at 100 C., was transferred to the Roux bath at 37 C. for from 2 to 5 hours. The desired amount of sol was then added to the serum, which previously had been warmed to 37 C.

A comparison of the results of these early tests (Table 46) with those obtained by the sol-gel method (Table 49) will show the superiority of the latter procedure, in which the 0.25-c.c. lethal dose was attained in 5 mixtures after an incubation of only 15 minutes. Table 46 is a composite of 36 separate experiments, in each of which the 37 C. sol was added to 2 c.c. of rat serum. The mixture was then shaken, sometimes very gently and at other times very vigorously, after which it was incubated for the stated time. It was then centrifugated at 3000 revolutions for 5 minutes, and the clear fluid injected. These tests are grouped according to the amount of agar used. The 7 mixtures indicated in the first column correspond with those heretofore used (Table 23), and to the sol-gel mixtures to be discussed later. The ratio is that of the agar solution to the serum.

It will be noted that 0.25 c.c. proved fatal in 2 instances (6a and 7), and then only after prolonged incubation. Tests 4 to 7 were made with the same pooled serum. Apparently, the best mixture is 6a, since this on incubation for 71½ minutes became fatal in dose of 0.5 c.c.; when incubated for 15 minutes it was nearly fatal in dose of 0.25 c.c.

The very severe result with mixture 9 is of interest as showing that an excess of agar does not interfere with poison-production. Nathan,¹ working with agar gel and guinea-pig serum, the mixture (0.5:1) corresponding with No. 8, did not succeed in producing a fatal anaphylatoxin, tho the amount injected was 4.5 c.c.

TABLE 46
ACTION OF AGAR SOL (37 C.) ON RAT SERUM
(MIXTURES KEPT AT 37 C., THEN CENTRIFUGATED AND TESTED)

Mixture		Guinea-Pig		Serum		Result
No.	Ratio	No.	Weight	B. C.* at 37 C.	c.c. (intra- venously)	
1	0.005:1	1	205	15 min.	1.0	Very slight
		2	250	1 hr.	"	Very slight
		3	173	2½ hr.	"	Very severe
		4	170	3 hr.	"	Slight
		5	175	6 hr.	"	3' 5"
		6	170	22 hr.	0.5	3'30"
		6a	170	22 hr.	0.25	1½ hr.
4	0.025:1	7	170	29 hr.	0.25	3'50"
		8	175	7½ min.	1.0	Very slight
		9	170	15 min.	1.0	3'40"
		10	202	30 min.	0.5	Fair
		11	225	1 hr.	1.0	3'40"
5	0.05:1	12	218	1 hr.	1.0	2'50"
		13	240	7½ min.	0.5	Very slight
		14	240	7½ min.	0.5	Very slight
		15	206	15 min.	0.5	Moderate
		16	227	15 min.	1.0	9 hr.
		17	197	30 min.	1.0	3'40"
6a	0.125:1	18	220	1 hr.	1.0	Severe
		19	220	7½ min.	0.5	3'50"
		20	173	7½ min.	0.5	3'15"
		21	177	15 min.	0.25	Near-kill
7	0.25:1	22	180	1 hr.	0.5	3'55"
		23	225	2½ min.	0.5	Very slight
		24	229	7½ min.	"	Very slight
		25	220	7½ min.	"	Slight
		26	215	7½ min.	"	Very slight
		27	175	15 min.	"	3'30"
		28	180	15 min.	"	3'40"
		29	240	1 hr.	"	6'20"
		30	210	1 hr.	"	Very severe
		31	245	1 hr.	"	Very slight
		32	175	2¼ hr.	"	3'35"
		32a	170	4 hr.	"	4'20"
8	0.5:1	33	220	15 min.	1.0	4'
9	1:1	34	196	15 min.	0.6	Near-kill

* B. C. = before centrifugation.

EXPERIMENTS WITH AGAR GEL

It has been pointed out that Bordet allowed the sterilized agar to gel over night, after which it was thoroughly shaken till it became a thick fluid; 1 volume of this softened agar was then added to 5 volumes

¹ Ztschr. f. Immunitätsf., 1913, 17, p. 480.

of serum, and the whole mixed and incubated at 37 C. for 2 hours; whereupon the mixture was centrifugated and the clear serum injected in dose of 4 to 5 c.c. Inasmuch as the previous work with trypanosomes had shown that rat serum could be rendered toxic in dose of 1 c.c. or less, by an incubation of only 15 minutes, it was felt that a like result could be obtained with agar. Accordingly a mixture of 1 part of shaken agar and 4 parts of serum was prepared and tested. As expected, after incubation for 1 hour the treated serum produced the typical fatal shock in dose of 1 c.c. Further tests soon showed that not only could this toxic dose be obtained after incubation for 15 minutes, but likewise the minimal lethal dose of 0.25 c.c. This fact was utilized as the basis of an extensive series of tests having for their object the demonstration of the presence of a ferment (anaphylase). It was soon found that the production of a serum of maximal toxicity was more an accident than a certainty, and this led to a long study of the underlying conditions.

As a rule, no difficulty was experienced in obtaining a toxic serum the lethal dose of which was 1 or even 0.5 c.c. This result was secured equally well after incubation for 7½ or for 15 minutes, and, moreover, a serum gave as good results after 24 hours as when perfectly fresh. It seemed, however, that much could be learned by determining the conditions which favored maximal toxicity.

At the outset it was desirable to ascertain whether the mode of sterilization in any way affected the findings.

For this purpose, a number of agar solutions were prepared by heating the agar with distilled water (0.5 gm. + 100 c.c.) in an autoclave at different temperatures. After sterilization the flasks of agar solution were either kept in the room or were placed in snow or ice to gel. The gel was then broken up by shaking and 1 part of the softened mass was then added to 4 parts of the rat serum and the mixtures vigorously shaken for 1 minute; they were then placed at 37 C. for the desired length of time, after which they were centrifugated at 3000 revolutions for 4 minutes, and the clear serum then injected. The results of these tests are given in Table 47.

With reference to these tests it may be stated that the same pooled serum was employed in the work with the 102, 110, and 120 C. agar; another pool, previously iced for 11 hours, was used with the 130 C. agar; that used with the 140 C. agar had been iced for 23 hours.

While this table would seem to show that the agar sterilized at 130 C. is more active than the others, as a matter of fact such conclusion is not justified, since the results are not constant. Frequently, the 130 C. agar failed to render a serum toxic in dose of 0.25 or even 0.5 c.c. On the other hand, the 120 C. agar seemed to be more certain in its action and for that reason was employed in most of the subsequent work. It may be added that the 110 C. agar, under like conditions, usually rendered serum toxic in dose of 0.5 c.c. in 7½ minutes.

Taken by and large, it is evident that the heat of sterilization, as employed, does not materially affect the inducing power of the agar.

In harmony with all similar observations is the result with Test 13 of Table 47, which shows that mere contact of agar and serum is not sufficient to produce anaphylatoxin. A like mixture placed in cracked ice for 1 hour and then tested was equally negative. Time and a moderate temperature are necessary to develop the reaction. At 37 C. even 2½ minutes (No. 14) are sufficient to toxify 1 c.c.; while 7½ minutes may be quite enough to toxify 0.25 c.c.

TABLE 47
EFFECT OF STERILIZATION UPON THE TOXIFYING POWER OF AGAR GEL

Sterilization of Agar		Guinea-Pig		Serum		Result
C.	min.	No.	Weight	B. C. at 37 C. (min.)	c.c. (intra- venously)	
102	15	1	180	7½	0.5	5'20"
		2	198	7½	0.25	3 hr.
		3	182	15	0.5	3'30"
		4	179	15	0.25	Fair shock
110	60	5	205	7½	1.0	3'35"
		6	225	7½	0.5	Fair
		7	235	15	0.5	3'25"
		8	185	15	0.25	Near-kill
120	30	9	178	7½	0.5	4'25"
		10	175	7½	0.25	Slight
		11	203	15	0.5	2'30"
		12	202	15	0.25	Slight
130	30	13	225	0	1.0	Slight
		14	170	2½	1.0	3'5"
		15	225	2½	0.5	Severe
		16	225	7½	0.5	3'50"
		17	170	7½	0.25	3'30"
		18	170	15	0.5	4'10"
		19	178	15	0.25	5'15"
		20	180	15	0.13	Slight
140	30	21	170	7½	0.5	3'10"
		22	172	7½	0.25	5½ hr.
		23	190	15	0.5	3'35"
		24	183	15	0.25	Slight

These facts are of much importance, being in line with many other observations all of which go to show that under favorable conditions anaphylatoxin-production occurs at great speed. The speed of production with agar is the same as that with trypanosomes, both being able to toxify rat serum in 2½ minutes so that it will kill in dose of 1 c.c.

In 2 instances (Nos. 2 and 22), it will be noted that 0.25 c.c. produced a severe shock followed by a subacute death; the initial shock was succeeded by severe prostration, paralysis of the extremities, and a progressive and rapid fall of temperature to 26 C. In general it may

be said that the more severe the shock, the more rapid the recovery. The subacute deaths from the rat anaphylatoxin are most unusual, tho they are common enough with rabbit serum.

As pointed out the results obtained with the 130 C. agar, as given in Table 47, appeared to show that such agar was the most active. Accordingly, this material was used in a large number of experiments, but with most discouraging results. With the utmost care in duplicating conditions, the results varied; the treated sera would sometimes fail to kill in dose of 1 c.c.; often they failed with 0.5 c.c., and it seemed almost impossible to obtain again the 0.25-c.c. lethal dose. In the course of 3 weeks not less than 10 different lots of 130 C. agar were tested. The conclusion was finally reached that apart from variations in the different lots of serum, an essential factor was a fugacious state of the agar gel.

Accordingly, and in order to secure the utmost uniformity, in subsequent experiments the agar was kept always in small Erlenmeyer flasks of 150-c.c. capacity or less. Immediately before use it was liquefied by heating in a water bath at 100 C. for 15 minutes, after which it was placed to gel in cracked ice (0 C.) for 1 hour. Icing for a longer period was found to be undesirable as it resulted in a harder gel that was more difficult to break up in the subsequent operations. The solid agar gel was then measured out directly by means of a pipet, which was cut off at the lowest graduation line, or the gel was first broken up by moderate swinging for 20 seconds. Such agar was then added to the previously iced serum and mixed either by tapping, or by swinging for 1 minute, or by means of a finely drawn-out pipet. The mixture thus prepared was now incubated, centrifugated, and tested.

Adherence to these conditions gave most excellent results, as will be seen from Table 48, in which are presented tests made on 3 consecutive days.

Fourteen different mixtures, each containing 2 c.c. of serum, were employed. For Nos. 1 to 4 the ratio of agar to serum was 0.125:1, while for the others it was 0.25:1. The agar employed for Nos. 13 and 14 was chilled at 0 C.; that for the others was gelled at -2 C., in a freezing mixture consisting of 1 part of salt and 50 parts of cracked ice. For Tests 5 and 9 to 12, the gel was not shaken up but was transferred to the serum as a solid cylinder; for the other tests it was thoroughly shaken for about 20 seconds. After the addition of the agar each mixture was shaken vigorously for 1 minute, except Test 13, which was given 2 minutes. The result of this test compared with that of No. 14, the same serum being used, appears to indicate that a slight change in the manipulation can affect the outcome. It is more likely, however, in view of the results shown in Table 45 that the resistance of the recipient was greater than usual.

Of the 5 different agars used in these experiments, those employed for Tests 9 and 12 were sterilized at 120 C. for 5 minutes, whereas the others were given 130 C. It is to be noted that the mixtures used for

Tests 1 to 4, which contain one-half the amount of agar present in those of Nos. 5 to 8, yield as good, if not better, results, tho the same pooled serum was used for all. It will also be seen that incubation at 37 C. for 7½ minutes may yield a lethal dose of 0.25 c.c. (No. 14), whereas 15 minutes sufficed in all the tests except in No. 13, which has been referred to. In test 10a a very severe shock was produced by as small a dose as 0.13 c.c.

TABLE 48

ACTION OF AGAR GEL (0 C.) ON RAT SERUM (0 C.)
(MIXTURES KEPT AT 37 C., THEN CENTRIFUGED AND TESTED)

Mixture		Guinea-Pig		Serum		Result
No.	Ratio	No.	Weight	B. C. at 37 C. (min.)	c.c. (intra- venously)	
6a	0.125:1	1	180	7½	0.25	Severe
		2	185	15	"	4' 5"
		3	200	30	"	2' 25"
		4	175	60	"	2' 40"
7	0.25:1	5	200	15	"	8' 30"
		6	170	"	"	3' 25"
		7	200	30	0.5	Slight
		8	170	60	0.25	Very slight
		8a	210	"	0.5	2' 55"
		9	185	15	0.25	2' 50"
		10	170	"	0.15	2' 20"
		10a	178	"	0.13	Very severe
		11	185	"	0.25	4' 55"
		12	181	"	"	3' 20"
		13	182	7½	"	Slight
		14	185	"	"	3' 50"

In experiments of this type the results depend very largely on the extent to which the agar is broken up in the process of shaking the mixture. With a moderate shake, many large lumps of agar, 2 mm. or more in diameter, can be seen in the serum. This is especially likely to occur when the gel is used at once after it has set at 0 C. Inasmuch as the fineness of the division cannot be controlled, it follows that the results by this method are not always as favorable as those given in Table 48. The general conviction arrived at after many trials was that the agar should be in the form of a hard gel, and thoroughly broken up while in that state; a transition toward the sol form apparently weakens the reaction. Excessive shaking of the gel before use, or a long standing of such shaken agar, impairs its efficiency.

EXPERIMENTS WITH SOL-GEL AGAR

It has been shown that it is possible to obtain in a few minutes by means of the agar gel a toxic serum such that 0.25 c.c. is acutely fatal. The method, however, did not yield this lethal dose with as much regu-

larity as was desirable, largely because of the difficulty of securing perfect distribution of the agar through the serum.

This difficulty was eventually overcome by devising the sol-gel method (Part II), in which as previously stated, the desired amount of sol at 37 C. is added to the serum, previously warmed to 37 C., and thoroughly shaken for 1 minute; after which the mixture is placed at once in cracked ice for 1 hour; it is then transferred to the Roux water bath at 37 C., and at intervals a portion is removed, centrifugated at 3000 revolutions for 4 minutes or more, and injected.

The results obtained by this procedure are superior to those obtained by either of the other methods, but it must not be inferred that they are strictly constant. A factor which is not controllable is the quality of the serum; even more important is the variation in the resistance of the guinea-pigs.

TABLE 49
ACTION OF AGAR-SOL-GEL ON RAT SERUM
(MIXTURES ICED FOR 1 HOUR, THEN INCUBATED AND CENTRIFUGATED)

Mixture		Guinea-Pig		Serum		Result
No.	Ratio	No.	Weight	B. C. at 37 C. (hr.)	c.c. (intra- venously)	
1	0.0005:1	1	220	1	1.0	Nil
		2	215	3	"	Nil
		3	215	6	"	Nil
		4	205	12	"	3/25"
3	0.005:1	5	190	1/4	0.25	4/45"
4	0.025:1	6	190	"	"	3/30"
5	0.05 :1	7	185	"	"	3/45"
6	0.1 :1	8	198	"	"	3/30"
7	0.25 :1	9	205	"	"	4'
8	0.5 :1	10	200	"	"	Slight
		10a	220	1	"	Very slight
9	1 :1	11	207	1/4	"	Very slight
		11a	212	1	"	Slight

In Table 49 will be found the results of experiments with several sol-gel mixtures which correspond to those employed with guinea-pig and rabbit serum. For the composition of these mixtures reference should be made to Table 23. Mixture 2 was not tested with rat serum.

It will be seen that Mixtures 3 to 7 toxified the rat serum at 37 C. within 15 minutes so that 0.25 c.c. was fatal. Noteworthy is the fact that Mixture 1, after incubation for 12 hours, rendered the serum toxic in dose of 1 c.c. The ratio of dry agar to this amount of serum is 1:400,000, or $\frac{1}{400}$ of the amount employed by Bordet in his work with

guinea-pig serum. The difference becomes more striking when it is remembered that Bordet injected from 4 to 5 c.c., and that Nathan's lowest fatal dose was 3 c.c. It should be pointed out, further, that the absolute amount of agar employed to toxify 1 c.c. of serum in this mixture was 0.0025 mg., while with Mixture 3, of which the lethal dose was 0.25 c.c., the corresponding amount of agar was only 0.00625 mg. — a *reductio ad absurdum* of Friedberger's hypothesis that the anaphylatoxin is derived from the cleavage of the nitrogenous constituents of agar. An absolute demonstration of its untenability will be given later when it will be shown that the mere addition of distilled water to rat serum results in the production of anaphylatoxin.

With Mixtures 8 and 9 the results were apparently poor, thus giving support to the view that an excess of agar interferes with the reaction. It must be noted, however, that the large amount of agar serves to dilute the serum, and that consequently the dose of 0.25 c.c. does not contain the full amount of serum constituents. In another experiment with Mixture 8, tests with 0.25 c.c. being made at intervals of 15 minutes, good shock effects were obtained at the quarter- and half-hour tests; while the test at 1 hour caused death in 60 minutes. Similarly, another No.-9 mixture gave good shocks at $\frac{1}{4}$, 1, $1\frac{1}{4}$, and $1\frac{1}{2}$ hours; without doubt 0.5 or 1 c.c. of these sera would have proved fatal, since, as shown in Table 46, the No.-8 sol-serum mixture did kill in dose of 1 c.c., while Mixture 9 in smaller dose almost proved fatal.

Mixture 6 was used in practically all the subsequent work with rat serum, and while it usually gave, in 15 minutes, a serum which was toxic in dose of 0.25 c.c., it failed to do so on several occasions. Such sera, however, were toxic when retested at the half or three-quarter hour period, tho in one instance the mixture had to be incubated for 1 hour before it killed in this dose. Similar variations must therefore be expected with the other mixtures for reasons already pointed out.

In view of the fact that rat serum can be toxified within 15 minutes so that 0.25 c.c. is a lethal dose, it will be evident that contact for a minute or two should be quite sufficient to render 2 c.c. fatally toxic. No effort was made actually to demonstrate this speed of poison-production with sol-gel mixtures. With the plain gel-serum mixtures, however, it was repeatedly found that $7\frac{1}{2}$ minutes at 37 C. sufficed to toxify 0.25 c.c., while even $2\frac{1}{2}$ minutes was enough to make 1 c.c. fatally toxic (Table 47). These facts bring out clearly the striking difference between the serum of the rat and that of the guinea-pig or rabbit.

APPARENT VARIATIONS IN TOXICITY

In the preceding work with trypanosomal anaphylatoxin, as well as with that obtained by the action of agar on guinea-pig and rabbit serum, it was shown that a treated serum was not uniformly toxic, but seemed to have an oscillation or periodicity. It was accordingly desirable to ascertain whether toxic rat serum exhibited the same peculiarity. Indications of such behavior had been frequently met with in working with this serum, but, in line with prevailing views, it was believed that the failure of a toxic serum to kill on a second injection, made some time after the first fatal test, was evidence of destruction of the poison. Serial injections to test this point were not made until it became possible to obtain a serum of maximal toxicity.

A preliminary series of tests was made with a No.-7 sol-gel mixture. This was iced for 1 hour, then incubated for 20 minutes at 38 C., after which it was centrifugated at 3000 revolutions for 10 minutes. The clear serum was tested at once, the balance being placed at 38 C. and tested at intervals of 15 minutes. With 0.25 c.c. as a dose, a severe shock was obtained in the immediate test and also at 15 minutes. The tests made at $\frac{1}{2}$, $\frac{3}{4}$, 1, $1\frac{1}{4}$, and $1\frac{1}{2}$ hours proved fatal, while that at $1\frac{3}{4}$ hour gave a near-kill. The deaths were all acute ($3\frac{1}{2}$ to $4\frac{1}{2}$ minutes), except in the three-quarter-hour test, in which it was protracted to 36 minutes. In the dose employed this serum was quite uniformly toxic, 5 out of 8 animals dying.

For the second trial, a No.-6 mixture was prepared in the same way as the preceding. The test made at once after centrifugation showed that 0.25 c.c. was fatal. Accordingly, the subsequent tests, at intervals of 15 minutes, were made with 0.2 c.c. The results of this experiment are given in Table 50 and are shown also in Chart 8. The fatal results in this experiment, it will be seen, were not consecutive and this variation might very well be considered as indicating an oscillation in the toxicity. Of 8 tests 4 proved fatal (compare Table 17).

A third test was made with a gel-serum mixture (0.25:1) for the purpose of producing a less toxic serum. The test with 0.25 c.c. made at once after centrifugation gave but a slight shock; those made at $\frac{1}{4}$, $\frac{1}{2}$, and $1\frac{1}{4}$ hours killed, whereas the other tests up to $2\frac{1}{4}$ hours gave but slight or moderate effects. Hence, in this experiment of 10 tests but 3 proved fatal.

Here, as elsewhere, the question arose as to whether the variations were due to changes in the toxicity, or to unequal susceptibility on the part of the guinea-pigs. To assume the latter would be the easiest way to account for the irregularities, but, inasmuch as the matter was one of considerable importance, it was necessary to present experimental proof. This was done with rabbit serum, and the results have already been presented in Table 45. In view of those results it follows that the variations noted are to be interpreted as expressive of the indi-

vidual resistance of the guinea-pigs. The sera used in the first two experiments, in dose of 0.5 c.c., would probably have been uniformly fatal. It is to be noted that in these experiments the poison persisted for the duration of the experiment, nearly 2 hours.

TABLE 50
APPARENT VARIATION IN TOXICITY OF RAT SERUM TREATED WITH AGAR
(SOL-GEL MIXTURE 6, RATIO 0.1:1)

Guinea-Pig		Serum		Result
No.	Weight	A. C.* at 37 C. (hr.)	c.c. (Intravenously)	
1	190	0	0.25	3:55"
2	206	1/4	0.20	Fair shock
3	170	1/2	"	3:55"
4	171	3/4	"	Moderate
5	173	1	"	Fair
6	182	1 1/4	"	3:10"
7	170	1 1/2	"	Severe
8	173	1 3/4	"	4"

* A. C. = after centrifugation.

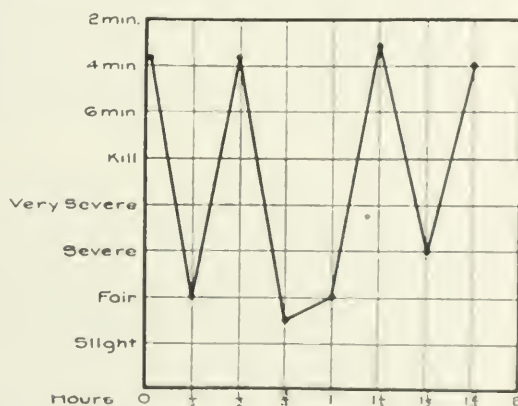


Chart 8. Apparent variation in toxicity of rat serum treated with agar (Table 50).

ATTEMPTED DEMONSTRATION OF FERMENT ACTION

Serial Dilutions.—The observation that mixtures of agar and serum which had not been incubated, even tho they had been iced for an hour, showed no immediate toxicity, suggested that possibly adsorption of an antagonistic body might occur at 0 C., thereby setting free the ferment, which then would require only a suitable temperature to act. Were this the case, high dilutions of such treated and centrifugated serum with normal serum should toxify readily. Acting on this theory, a large number of experiments were made with iced mixtures, having

for their purpose the demonstration of the presence of a ferment (anaphylase). The very first experiment of this kind gave a most surprising result: even an E dilution, representing $\frac{1}{256}$ of the original treated serum, rendered 1 c.c. toxic after an incubation of $2\frac{1}{2}$ hours. Two of these experiments in tabulated form are sufficient to illustrate the results obtained.*

The method of procedure was essentially the same as that employed in the corresponding tests with guinea-pig serum (Table 30). In the first experiment, the results of which are given in Table 51, 1.5 c.c. of agar gel were added to 6 c.c. of a pooled rat serum, both being at room temperature. The mixture was at once placed in ice and thoroughly shaken for 1 minute. It was then kept at 0 C. for 1 hour, after which it was transferred to iced tubes and centrifugated at 3000 revolutions for 6 minutes. A portion of this serum, designated as A, was tested 9 minutes later (No. 1). Another portion was placed at 37 C. and tested after 1 hour (Nos. 2 and 3). Another portion was used to make the dilution series. For the latter, 1 c.c. of A was added to 3 c.c. of the pooled serum, giving a 1:4 dilution, or B. Diluting 1 c.c. of B in like manner gave the 1:16 dilution C, and this process was repeated yielding the 1:64 dilution D, and then the 1:256 dilution E. For a control, another portion of A was placed at 60 C. for one-half hour, to destroy any ferment that might be present. The control B dilution was made by adding 1 c.c. of this heated serum to 3 c.c. of the pooled serum. These dilutions, in small Erlenmeyer flasks, were placed in a Roux water bath at 37 C., and tested at the times indicated. The same pooled serum was employed for all these tests, and it is hardly necessary to add that strictly sterile conditions obtained.

For the experiment given in Table 52, 1 c.c. of gel and 4 c.c. of rat serum, both previously kept at 0 C. for 1 hour, were mixed and thoroughly shaken for 1 minute, after which the mixture was placed in ice for 1 hour. It was then transferred to iced tubes and centrifugated at 3000 revolutions for 4 minutes. The supernatant serum, designated as A, was then used to make dilutions B, C, D, and E, the same procedure being followed as given in the foregoing.

For a control, 4 c.c. of the same pooled normal serum were heated at 60 C. for 1 hour, to destroy any ferment that might be present. This inactivated serum, cooled to 0 C., was treated with agar gel at the same time as the foregoing, the two tests running parallel. The control A thus obtained was then used to make the control dilutions (into normal serum) B, C, D, and E, which were then incubated and tested at the same time as the former. For all the tests in this experiment a single pooled serum was employed.

The results presented in Tables 51 and 52 are of interest inasmuch as, at first sight, they would tend to support the ferment theory. It will be seen that in both experiments, all 4 dilutions became toxic after a relatively short incubation. The B dilutions of both sets, it is to be noted, developed the lethal dose of 0.25 c.c.

The correct interpretation of these, and many similar experiments, centers about the inducing action of minute amounts of agar for, it is to be assumed, some agar is left in the serum after centrifugation at

3000 revolutions for 4 or 6 minutes, as was done in the foregoing. Particles of agar which have swollen by imbibition, may at 0 C. possess a density approximately that of serum, and consequently such floaters

TABLE 51
I. APPARENT FERMENT ACTION
(PROGRESSIVE DILUTION OF TREATED RAT SERUM WITH NORMAL SERUM)

Series	Guinea-Pig		Serum			Result
	No.	Weight	Dilution	Hours at 37 C.	c.c. (intravenously)	
A	1	187		0	1.0	3'20"
	2	185		1	0.5	2'45"
	3	175		1	0.25	Slight
B	4	198	1:4	1½	1.0	3'
	5	170	1:4	1½	0.5	3'10"
	6	185	1:4	1½	0.25	2'20"
C	7	170	1:16	1¾	1.0	3'20"
	8	170	1:16	1¾	0.5	Very severe
D	9	172	1:64	2	1.0	4'5"
	10	205	1:64	2	0.5	Slight
E	11	170	1:256	2½	1.0	3'
	12	190	1:256	2½	0.5	Very slight
Control B	13	240	1:4	1½	1.0	3'
	14	182	1:4	1½	0.5	Very severe

TABLE 52
II. APPARENT FERMENT ACTION
(PROGRESSIVE DILUTION OF TREATED RAT SERUM WITH NORMAL SERUM)

Series	Guinea-Pig		Serum			Result
	No.	Weight	Dilution	Hours at 37 C.	c.c. (intravenously)	
B	1	177	1:4	2	0.5	2'55"
	2	170	1:4	2	0.25	4'20"
C	3	180	1:16	2¼	0.5	4'15"
D	4	201	1:64	2½	1.0	5'
E	5	203	1:256	2½	1.0	2'40"
	6	200	1:256	2¾	0.5	3'20"
Control B	7	211	1:4	2	1.0	3'35"
Control C	8	205	1:16	2¼	1.0	5'25"
Control D	9	175	1:64	2½	1.0	3'50"
Control E	10	208	1:256	2¾	1.0	Slight

will resist the centrifugal force. This is especially true of the inactivated serum, which may retain in suspension all of the agar. This is what actually occurred in the control test in the experiment given in

Table 52. The serum which was inactivated at 60 C. for 1 hour, then treated with agar as has been indicated, gave no visible deposit after centrifugation for 4 minutes. It was consequently centrifugated again for a like period, but even then scarcely any agar was thrown down. Similar mixtures of gel and inactivated serum, when centrifugated at 8000 revolutions yielded an agar deposit, and the supernatant fluid in dilution C gave a severe shock after incubation for $2\frac{1}{4}$ hours. Dilutions D and E showed no effect. The control dilutions B, C, D, and E were made with this inactivated serum, which contained, therefore, practically all of the original agar. It will be observed from the table that the control dilutions B, C, and D (Nos. 7 to 9) became toxic.

If it is assumed that the hypothetical ferment was destroyed by heating to 60 C. for 1 hour, it follows that the effects produced by these dilutions, which were made into normal serum, were due to the transferred agar. And, since the ratio of agar to serum in the original mixture was 1:800, it follows that the ratios in the control dilutions B, C, D, and E were 1:3200; 1:12800; 1:51200; and 1:204,800, respectively.

The necessity of bearing in mind the action of minute amounts of agar will be fully realized when reference is made to the work with sol-gel mixtures (Table 49). It will be seen there that Mixture 1, having an agar-serum ratio of 1:400,000, toxified 1 c.c. of the serum, the test being made after incubation for 12 hours; while Mixture 3, the ratio in which is 1:40000, after only 15 minutes' incubation gave a lethal dose of 0.25 c.c. In view of these facts it is evident that the action of the residual agar must not be overlooked. The incompleteness of the precipitation of the agar at 3000 revolutions is demonstrable by resubmitting the serum to a higher speed, as has been pointed out in Part II. The effects of such treatment on the inducing power will be presently discussed.

Similarly, the control B dilution of Table 51, made with a portion of Serum A which had been heated at 60 C. for half an hour, became toxic, tho the amount of agar present in this instance was much less than in those mentioned above, since the inactivation took place after centrifugation for 6 minutes at 3000 revolutions. Unless it be assumed that the ferment is not destroyed by heating at 60 C., it follows that the toxicity is due either to the action of the residual agar or to that of the heated serum.

Effect of Centrifugation.—Besides the controls with heated sera,

referred to, direct attempts were made to determine the action of the residual agar in mixtures which had been centrifugated for different lengths of time, or at varying speed. One of these experiments, reproduced in Table 53, is reasonable evidence that the inducing power of a treated serum is due to the retained agar.

In this, as in the preceding experiment, the mixture of 1.5 c.c. gel and 6 c.c. of rat serum, both previously kept at 0 C. for 1 hour, was vigorously shaken for 1 minute, then placed in cracked ice for 1 hour. It was then transferred to iced tubes and centrifugated at 3000 revolutions. At the end of 2 minutes, the machine was stopped and a portion of the supernatant fluid was used to make the first series of dilutions, B, C, D, and E (Exper. 1). At this time the agar was incompletely thrown, as shown by the small deposit and by the presence of floaters; consequently this series should have become readily toxified, and such actually took place.

After the removal of the portion needed for this series of dilutions, the centrifugation of the remainder was continued for 18 minutes more, making a total of 20 minutes. As the otherwise clear supernatant serum showed about 8 large floaters, it was again centrifugated for 1 minute but without throwing the latter; a further swing for 2 minutes likewise failed. The serum with the floaters was then decanted and again centrifugated for 2 minutes with no better result. In order to avoid further delay, the remaining floaters were fished out with a platinum wire, and the clear serum was then used for the second series of dilutions (Exper. 2). It will be noted that the serum for this second series was centrifugated for 25 minutes, whereas that for the first was given but 2 minutes. The same pooled serum was used for both series of tests.

As might well be expected, Table 53 shows that the toxifying power of a treated serum is greatly reduced by an increased centrifugation. The fact that visible floaters persisted in such serum even after centrifugation for 25 minutes, makes it probable that finer particles of agar were still present, and that the comparatively slight effect obtained in the second series was due to this residuum.

As further evidence of the effect of centrifugation, it may be mentioned that several gel-serum mixtures thus treated, were centrifugated at 3000 revolutions and portions of the supernatant fluid removed at 2, 6, 12, and 24 minutes; these were then incubated at 37 C. for 3 hours and tested, the dose being 0.5 c.c.. In one trial, the first portion was fatal, while the others showed progressively less and less effect; in another trial the first and second portions produced severe shocks, while the third and fourth gave no effect. This gradation in effects is clearly due to the decrease in the amount of residual agar by the continued centrifugation.

Of the many other efforts to determine the point at issue, perhaps a few will serve as confirmatory of the preceding. The comparative effect of centrifugation at different rates of speed will be seen in the 3 experiments given in Table 54. For Exper. 1, a mixture of 1.5 c.c. of gel and 6 c.c. of distilled water, both previously kept at 0 C., was shaken as usual, then iced

for 1 hour, after which it was centrifugated at 3000 revolutions for 4 minutes. The clear supernatant water was then used to make dilutions B, C, and D.

For Exper. 2, an exactly similar mixture was made, and, after icing for 1 hour, it was centrifugated at 8000 revolutions for 20 minutes. The clear supernatant water was then used to make the dilutions B, C, and D of that series.

It was expected that these two series of tests would show the effect of any residual agar left in suspension in distilled water, after centrifugation at 3000 and 8000 revolutions, respectively; that in the case of the latter the amount of retained agar would be less with corre-

TABLE 53
COMPARATIVE EFFECT OF CENTRIFUGATION FOR 2 AND 25 MIN. AT 3000 R.P.M.
(APPLIED TO A GEL-SERUM MIXTURE, RATIO 0.25:1, ICED 1 Hr.)

Series	Guinea-Pig		Serum			Result
	Hr.	Weight	Dilution	Hours at 37 C.	c.c. (intra-venously)	
Exper. 1*						
B	1	205	1:4	2½	1.0	3'40"
C	2	180	1:16	"	"	3'50"
D	3	204	1:64	2¾	"	2'55"
E	4	207	1:256	"	"	Severe
	4a	200	1:256	9	"	3'45
Exper. 2*						
B	5	205	1:4	3¼	1.0	4'15"
C	6	205	1:16	"	"	Very slight
	6a	200	1:16	9	"	Severe
D	7	176	1:64	3¼	"	Slight
	7a	200	1:64	9	"	Very slight
E	8	202	1:256	3¼	"	Very slight
	8a	200	1:256	9	"	Nil

* The dilutions of Exper. 1 were made after centrifugation for 2 minutes; those of Exper. 2 after centrifugation of the same mixture for 25 minutes.

sponding decrease in the inducing power. When the results of Exper. 2 are compared with those of Exper. 1, this is found to be the case. The very slight effects obtained with dilutions B, C, and D of Exper. 2 are really no more than would have been obtained with a like dose of normal serum incubated for the same length of time. Consequently, Exper. 2 is to be considered as showing that at 8000 revolutions practically all the agar is thrown out of an agar-water suspension. A similar suspension centrifugated at 3000 revolutions for 4 minutes is

not freed of its agar and hence the results seen in Exper. 1. Incidentally, a comparison of the latter results with those of Table 52, where an agar-serum mixture is shown to have been centrifugated at like speed for the same length of time, shows that the denser serum probably holds up more of the agar than does the water. This is even more certain when Expers. 2 and 3 of Table 54 are compared.

For Exper. 3 a gel-serum mixture was prepared and treated in exactly the same way as for the experiment recorded in Table 52, except that it was centrifugated at 8000 revolutions for 20 minutes. A comparison of the results in Expers. 2 and 3, both mixtures having been centrifugated at the same high speed and for the same length of time, shows that the serum mixture possesses some inducing power which is not present in the agar-water mixture; in other words, the agar is not removed as completely from the serum as it is from the water suspension. A further comparison of the results obtained in Exper. 3 with those of Table 52, both being from serum mixtures but mixtures subjected to different rates of centrifugation, shows at once that the high speed enormously reduces the inducing power of a treated serum. It is reasonable to believe that centrifugation at 8000 revolutions for an hour would still further decrease this toxifying power.

It is not to be assumed that the results obtained after centrifugation at 8000 revolutions for 20 minutes (Table 54) are always equally favorable. It has occurred in similar experiments that the B and C dilutions did kill in a 1-c.c. dose, after incubation for $2\frac{1}{4}$ hours. Indeed, in one such experiment the B and C dilutions killed in dose of 0.5 c.c. In another experiment in which the centrifugation lasted but 15 minutes, even the dilution D killed after being incubated for 8 hours. Results of this kind, occurring after long incubation, must be regarded with suspicion owing to the liability of normal rat serum to become toxic through mere incubation.

With recognition of the evident fact that centrifugation does reduce the toxifying power of a treated serum, it can be assumed that this is due to the removal not merely of agar, but rather of a suspenoid form of the ferment. It is possible to conceive that the finely divided agar adsorbs the ferment and that the latter while in this state exerts its characteristic action. If such were the case, obviously, centrifugation would remove the ferment-laden agar, thereby lessening the toxifying power of the serum. It was necessary, therefore, to test this possibility by direct experimentation; and first of all, it had to be shown that the

mere process of centrifugation did not of itself destroy the activity of such suspended ferment.

When a gel-serum mixture, after icing for 1 hour, is centrifugated at 3000 revolutions for 6 minutes, and the resulting apparently clear serum is then recentrifugated at 8000 revolutions for 20 minutes, it

TABLE 54
COMPARATIVE EFFECT OF CENTRIFUGATION AT 3000 AND 8000 R.P.M.

Series	Guinea-Pig		Serum			Result •
	Hr	Weight	Dilution	Hours at 37 C.	c.c. (intra- venously)	
Exper. 1*						
B	1	225	1:4	3	1	Moderate 5'10"
	1a	190	1:4	3½	"	
C	2	212	1:16	3¼	"	4'25"
D	3	210	1:64	3¼	"	Slight 3'40"
	3a	177	1:64	9	"	
Exper. 2*						
B	4	171	1:4	3	1	Nil Very slight
	4a	208	1:4	10½	"	
C	5	202	1:16	3¼	"	" "
	5a	202	1:16	10½	"	
D	6	171	1:64	3¼	"	" "
	6a	213	1:64	10½	"	
Exper. 3†						
B	7	177	1:4	3	1	Slight 2'25"
	7a	180	1:4	8½	"	
C	8	198	1:16	3	"	Very slight Very severe
	8a	230	1:16	8½	"	
D	9	185	1:64	3¼	"	Very slight Very slight
	9a	178	1:64	8¾	"	
E	10	190	1:256	3¼	"	Very slight

* In Exper. 1 a gel-water mixture was centrifugated at 3000 r.p.m. for 4 minutes and dilutions then were made. In Exper. 2 a like mixture was swung at 8000 r.p.m. for 20 minutes.

† In Exper. 3 a gel-serum was given 8000 r.p.m. for 20 minutes.

yields a slight deposit or film on the glass. In one experiment, the deposit and the supernatant serum were stirred up and again centrifugated at the high speed. The deposit and the serum having again been thoroughly stirred up, this mixture was used to make Dilutions B and C with normal serum. On incubation for 3½ hours, these dilu-

tions caused acute death in 0.5-c.c. dose, and behaved in that respect exactly the same as the corresponding dilutions made with a serum obtained after centrifugation at slow speed. This test, therefore, served to show that high-speed centrifugation did not destroy the inducing power of the residual agar, or of the supposed ferment, but that its action consisted merely in removing the suspended matter from the serum.

Heating of the Mixture to 60 C. before Incubation.—The fact that the deposit thrown down by centrifugation, on subsequent mixture with normal serum, gives rise to poison may mean nothing more than agar action, and yet to prove that such is the case is not easy. All attempts to do so were made on the assumption that the supposed ferment was destroyed by heating at 56 C. or 60 C., and this may not be justifiable. The inactivation of a normal serum by heat does effect a marked change in the serum, since it is no longer readily toxifiable with agar or with a treated serum. This is true even when the serum is heated for a short time at 50 C., as has been shown with rabbit serum (Part III). Further evidence of this will be supplied later.

It has been shown in Table 44 that an agar serum is apparently toxified at 50 C. A similar experiment at 60 C. with rat serum may be cited at this point. A gel-serum mixture after thorough shaking for 1 minute was divided into 2 portions. The first was placed at 37 C. for 15 minutes. When tested it was found to be fatal in a dose of 0.25 c.c. The second portion was placed at 60 C. for 1 hour, after which it was also incubated at 37 C. for 15 minutes. When tested, it gave a severe shock in dose of 1 c.c. The obvious conclusion was that some poison was made, but it would be wrong to assume that this occurred either at 60 C. or during the subsequent incubation. It is more likely, and such eventually was shown to be the case, that the poison was formed before the temperature rose over 50 C. in the first heating. Hence results of this kind cannot be used to support or invalidate the ferment conception.

The one conclusion which can be drawn from such experiments is that the poison is not destroyed at the temperature employed. The heating of a toxic serum at 60 C. for 1 hour does not alter its action. Incidentally, it may be added that in one experiment a toxic serum was kept at 56 C. and when tested at 3, 6, and 24 hours was found to be fatal in dose of 1 c.c. Prolonged exposure at this temperature seems, therefore, to have very little effect on the poison.

The rat anaphylatoxin is destroyed, however, at higher temperatures as will be seen from the following experiment. A toxic serum having the lethal dose of 0.25 c.c. was dialyzed in a collodium sac at 50 C. for 15 minutes; retested it was found to kill in dose of 0.5 c.c. serum equivalent, but not in 0.25 c.c. It was then divided into 2 portions: one was placed at 70 C. for half an hour, after which it was tested and found to be fatal in dose of 1 c.c. serum equivalent, but not in 0.5 c.c.; the other portion was heated in a water bath at 100 C. for 5 minutes, and when tested it was found to be without effect in dose of 2 c.c. serum equivalent. The higher the temperature, the more rapidly is the poison destroyed.

Action of Treated Serum on Heated Normal Serum.—In view of the fact that agar does not toxify an inactivated serum very readily, it is to be expected that a treated serum would have still less action unless the residual agar had acquired a marked activity. The following experiments will show that there is no reason to believe that this does occur.

A gel-serum mixture, after icing for 1 hour, was centrifugated at 3000 revolutions for 6 minutes; a portion of this treated serum (1 c.c.) was added to 3 c.c. of normal serum thus giving Dilution B; a like portion was added to 3 c.c. of the same pooled serum but inactivated at 60 C. for 1 hour, giving the dilution B'. Of these mixtures, after incubation at 37 C. for 1 hour, the former killed in dose of 1 c.c., while the latter showed no effect even after incubation for 2 hours.

In a similar experiment, in which the mixture, however, was centrifugated for only 2 minutes, the B and C dilutions of the treated serum with normal serum were fatal, even in dose of 0.5 c.c., after incubation for 2¼ hours; the corresponding dilutions with inactivated serum (60 C. for 1 hour) gave very severe shocks in the same time, thus showing that such serum can be toxified to some extent.

In another experiment, in which the dilutions B and C were made with a serum which had been heated to 56 C. for only half an hour, after incubation for 3 hours, the former killed in dose of 1 c.c., while the latter dilution gave a very severe shock. Since the treated serum when incubated alone may develop a lethal dose of 0.25 c.c., it follows that the effect of a B dilution with inactivated serum may be due to the original serum which is carried over into the dilutions.

These illustrations appear to show that a treated serum, obtained by low-speed centrifugation, is able at times to toxify a serum which has been heated to 56 C. and even to 60 C.; the explanation is probably that just given. This result is not obtained when high-speed centrifugation is employed, because of the more complete removal of the residual agar.

Action of the Agar Deposit on Heated Normal Serum.—In view of these results, it should be possible to toxify an inactivated serum by the addition of the agar deposit obtained by centrifugation, and experiments made with this object in view were successful.

In these tests, the gel-serum mixture after icing for 1 hour, and without incubation, was centrifugated at 8000 revolutions for 20 minutes. After the removal of the supernatant fluid, the deposit was taken up with 5 c.c. of serum, previously heated to 56 C. for 1 hour, and this mixture was then incubated at 37 C. In 1 trial, the test made at 3 hours with 1 c.c. gave a severe shock, and that at 8 hours killed in 2 minutes 10 seconds. A like result was obtained in a second experiment of this kind, while in a third fairly severe shocks were obtained. Of 2 other experiments, after incubation for 3 hours, one mixture caused death in 3 minutes 5 seconds, while the other produced a severe shock.

These results are fair evidence that the agar deposit is able to toxify a serum inactivated at 56 C. Whether this is due entirely to the agar, or to an adsorbed ferment, was a question which it was hoped would be settled by suitable control experiments, which were made at the same time as those mentioned. Mixtures of gel and distilled water, or salt solution, in place of normal serum, were iced for 1 hour, after which they were centrifugated and the agar deposit taken up with the inactivated serum and incubated at 37 C. The injections made at the end of 3 and 8 hours were practically negative, showing that the plain agar under these conditions could not toxify the heated serum. These results at first sight would seem to prove the presence of a ferment in the agar deposit obtained from a treated serum. There are, however, serious objections. In the first place, it will be shown that occasionally plain agar will toxify an inactivated serum, which fact of itself excludes the question of an adsorbed ferment. Again, it is possible that the agar from an iced serum mixture may adsorb or imbibe the matrix of the poison, which on subsequent incubation with inactive serum becomes toxified.

Attempts to Extract Ferment from the Agar Deposit.—If the agar adsorbed a ferment in some such way as fibrin is said to take up thrombin, it should be possible to extract it by means of distilled water or salt solution. A number of attempts to do this failed to give unequivocal results.

Thus, in one experiment a sol-serum mixture after incubation for 1 hour at 37 C., was centrifugated, and the deposit was then taken up with 4 c.c. of salt solution and digested at 37 C. for 1 hour. This suspension was now centrifugated and 1 c.c. of the extract was added to 3 c.c. of normal serum, and this mixture, kept at 37 C., when tested after 3 and 6 hours gave a fair shock.

Again, a mixture of 3.5 c.c. of gel and 14 c.c. of serum, after being iced for 1 hour, was centrifugated at 8000 revolutions for 20 minutes, and the agar deposit taken up with 14 c.c. of distilled water. This suspension after being digested at 37 C. for one-half hour was centrifugated the same as before; the extract added to a like volume of normal serum was incubated at 37 C. for 3½ hours and tested. The equivalent of 1 c.c. of serum, the usual dose, killed in 8 minutes.

This result seemed to indicate that a ferment had been extracted from the agar deposit. It was possible, however, that a small amount of agar was redissolved and that this toxified the serum. In order to control this point, a mixture of agar gel and distilled water was treated in exactly the same way as the gel-serum mixture. The final distilled-water extract when added to an equal volume of serum and incubated gave no effect at the 3-hour test, but at 8 hours gave a severe shock. Clearly, some agar had dissolved, tho apparently less than in the experiment proper. While this explanation may account for the result obtained, it must be borne in mind that distilled water itself tends to toxify rat serum.

When the digestion of the agar precipitate with distilled water was carried on at 45 C., the inducing power of the extract appeared to be higher than that obtained at 37 C., and similarly, digestion for 1 hour was better than one for 15 minutes. The effect of heat on this active extract will be seen from the following:

The final extract obtained by treating an agar deposit with distilled water at 45 C. for 1 hour was divided into 2 portions. One portion was added to an equal volume of normal serum, and the mixture, at 37 C., gave in 3 hours a severe shock, and in 7½ hours killed in 3 minutes. A second portion of the aqueous extract was heated to 100 C. for 3 minutes, then cooled and added to an equal volume of serum; the resultant mixture was incubated and tested at the same time as the preceding. This serum with the heated extract failed to give any appreciable effect at the 3- and 7-hour tests. The agar deposit used for this test was obtained from a gel-serum mixture which had been iced but not incubated.

The experiment just given might be taken to point to the presence of a ferment, but such interpretation must be questioned. The speed of the reaction is not such as it ought to be were a ferment actually concerned. The presence of minute amounts of agar in the extracts appears to be the best explanation for the results obtained.

Action of Heated Treated Serum on Normal Serum.—Another series of attempts to show whether the action of the residual agar was due to the agar itself or to an adsorbed ferment consisted in inactivating the treated serum obtained after the centrifugation of the iced gel-serum mixture. By heating such serum to 60 C. or 70 C. or even to 100 C. it should be possible to destroy the hypothetical ferment. The dilutions of the inactivated serum with normal serum (B and C) on subsequent incubation and testing might throw some light on the question.

One experiment of this kind is to be found in Table 51 (Nos. 13 and 14). It will be seen that in this case the serum which had been heated at 60 C. for 1 hour was capable of toxifying the B dilution. In another exactly similar experiment no effect was obtained when the serum was tested at the end of 1 hour; retested after 28 hours at 37 C. it proved fatal, but no special significance is to be attached to this result, since normal rat serum on prolonged incubation may become toxic in dose of 1 c.c. In a third experiment, the treated serum being likewise heated at 60 C. for 1 hour, the B dilution after incubation for 2 hours had a slight effect, while the C dilution tested at the same time produced a severe shock. The experiments with B and C dilutions of treated sera which had been heated to 70 C. and to 100 C. were entirely negative. In order to avoid coagulation when inactivating at high temperatures, the treated serum was first dialyzed in a collodium sac against distilled water, at 50 C. for about 5 minutes.

While these results might be taken to indicate the destruction of a ferment, the more reasonable explanation seems to be that the state of the residual agar is altered by the higher temperatures and becomes less active as an inducing agent.

Action of Agar on Heated Normal Serum.—According to Bordet, agar does not toxify guinea-pig serum which has been heated to 56 C., and a like conclusion was reached by Nathan, who worked with agar, starch, and inulin. This is undoubtedly true for the conditions under which they worked. Since guinea-pig serum does not readily yield as low a toxic dose as rat serum, it is clearly not as well adapted for the study of this reaction.

The work with trypanosomes (Table 14), however, showed that rat serum, the inactivated, could be rendered poisonous, and this fact led to similar tests with agar. The very first attempt with agar resulted favorably.

In this test, a mixture of 1 c.c. of agar gel and 4 c.c. of rat serum, previously heated to 56 C. for half an hour, when incubated at 37 C. for 15 minutes and then centrifugated gave a clear serum which in dose of 2 c.c. caused typical shock and death in 4 minutes 30 seconds. A control experiment made at the same time with normal serum gave a lethal dose of 0.25 c.c. In 5 other tests, made at different times, with 1 c.c. as the dose, little or no effect was obtained. It has been shown that the agar deposit after centrifugation of a gel-serum mixture can toxify an inactivated serum so that 1 c.c. is fatal.

It appeared from these tests that the inactivated serum was less easily toxified than normal serum, and that the toxicity secured was but a fraction of that which can be gotten with normal serum.

In the course of the work of the following year it was realized that the behavior of serum when heated was deserving of a more extended study. It will be shown in Part IX that even a short exposure at 50 C. alters the serum, and renders it less toxifiable by addition of distilled water. This observation led to similar tests with agar, which will be considered at this point.

Inactivation at 50 C.—It has been shown in connection with Table 44 that while agar apparently is able to toxify rabbit serum at 50 C., in reality the poison is produced before this temperature is reached. In view of this fact it was desirable to ascertain the action of agar on rat serum when incubated at 45 C., it being possible that this temperature was more favorable for poison-production than that of 37 C.

For this purpose a sol-gel serum mixture (No. 7) was prepared, the sol and serum being each warmed to 45 C. (the latter, for one-half hour) before mixing; it was then iced for 2 hours, after which it was placed at 45 C. and tested as indicated in Exper. 1, Table 55. It will be seen that under these conditions agar readily toxifies the serum, giving a lethal dose of 0.25 c.c. about as readily as at 37 C.

For Exper. 2, a portion of the same pooled serum, previously heated to 50 C. for half an hour, was treated with agar gel (1:4); after vigorous mixing for 1 minute, the mixture was placed at once at 45 C. and tests were made, in pairs, at 15-minute intervals, as shown in Table 55 (see Table 45).

A strict comparison of the two experiments is unfortunately impossible since in the former the sol-gel method was employed while in the latter the gel procedure was used. Nevertheless, it will be apparent that the serum which had been heated to 50 C. was less reactive than that exposed to 45 C. for a like time.

This experiment with a serum previously heated at 50 C. for half an hour was repeated on the following day, the method of procedure being exactly the same. It will be seen that altho the speed of the reaction appears to be less than usual, the results are very favorable, since even the lethal dose of 0.25 c.c. was obtained (Table 55, Exper. 8).

Inactivation at 56 C.—The results with serum inactivated at 56 C. showed an enormous decrease in capacity to produce anaphylatoxin. This will be apparent on comparison of Tables 55 and 56.

For Exper. 1 of Table 56, the serum was heated at 56 C. for half an hour; it was then treated with gel, as in the preceding test, after which the mixture was incubated at 45 C. and tested. The fact that 1 c.c. of this treated serum caused a good shock in 2 of the tests indicated that a fatal result could be expected with twice that dose. Accordingly, the experiment was repeated, tho with a different pooled serum which had been also heated to 56 C. for half an hour. This was used for Expers. 2 and 3 (Table 56), the former mixture being incubated at 45 C., the latter at 38 C. It will be seen

from the table that the treated inactivated serum, even in dose of 2 c.c., was only exceptionally toxic.

It was thought that possibly better results could be obtained by dialyzing the serum in a collodium sac at 55 C. for half an hour. In Exper. 4, made with this object in view, the volume of the serum was doubled in the course of the dialysis. The agar gel was then added to the dialyzed serum (1:4), thoroughly shaken, and the mixture placed at 38 C. The equivalent of 2 c.c.

TABLE 55

ACTION OF AGAR ON RAT SERUM WHICH HAS BEEN HEATED FOR HALF AN HOUR AT 45 C. (EXPER. 1); AND AT 50 C. (EXPER. 2 AND 3)

Guinea-Pig		Serum		Result
No.	Weight	Hours at 45 C.	c.c. (Intra- venously)	
Exper. 1*				
1	203	$\frac{1}{4}$	1.0	3' 5"
1a	202	$\frac{1}{4}$	1.0	3'40"
2	186	$\frac{1}{2}$	0.5	3'40"
2a	185	$\frac{1}{2}$	0.5	2'40"
3	190	$\frac{1}{2}$	0.25	3'30"
3a	190	$\frac{1}{2}$	0.25	Very near-kill
Exper. 2*				
1	204	$\frac{1}{4}$	1.0	Moderate
1a	207	$\frac{1}{4}$	1.0	3'40"
2	190	$\frac{1}{2}$	1.0	Moderate
2a	190	$\frac{1}{2}$	1.0	4'30"
3	195	$\frac{3}{4}$	1.0	4'15"
3a	190	$\frac{3}{4}$	1.0	4'
4	196	1	0.5	Slight
4a	196	1	0.5	Slight
Exper. 3*				
1	195	$\frac{1}{4}$	1.0	4'30"
1a	193	$\frac{1}{4}$	1.0	2'50"
2	188	$\frac{1}{2}$	0.5	Good
2a	187	$\frac{1}{2}$	0.5	Good
3	202	$\frac{3}{4}$	0.5	4'45"
3a	203	$\frac{3}{4}$	0.5	2'40"
4	178	1	0.25	3'45"
4a	175	1	0.25	Severe

* Duplicate inoculations were made at each interval as shown in table.

of serum was used as the dose. It will be seen that in this experiment the toxic effects were a trifle more marked than in the others.

This result led to further experiments with serum dialyzed at 50 C. for 15 minutes, then heated in a test tube at 56 C. for half an hour. The serum thus inactivated was then treated either by the gel or the sol-gel method. Of 3 such experiments, 2 gave little or no evidence of poison-production, while 1 developed a very severe shock after incubation for 30 minutes.

It will be seen from the foregoing, that while it is possible at times to toxify a rat serum which has been inactivated by heating at 56 C., more often the results are negative. Obviously, a larger dose than 2 c.c. might give more pronounced effects. As the matter stands, however, it is clear that the change inaugurated by heating at 50 C., becomes more complete by exposure to 56 C.

TABLE 56

ACTION OF AGAR ON RAT SERUM WHICH HAD BEEN HEATED FOR HALF AN HOUR AT 56 C.

Guinea-Pig		Serum		Result
No.	Weight	Hours at 45 C. or 38 C.	c.c. (Intravenously)	
Exper. 1*				
1	185	$\frac{1}{4}$	1	Moderate
1a	187	$\frac{1}{4}$	"	Severe
2	202	$\frac{1}{2}$	"	Slight
2a	205	$\frac{1}{2}$	"	Good
3	185	$\frac{3}{4}$	"	Very slight
Exper. 2*				
1	205	$\frac{1}{4}$	2	Very slight
1a	203	$\frac{1}{4}$	"	Moderate
2	198	$\frac{1}{2}$	"	Moderate
2a	202	$\frac{1}{2}$	"	Slight
Exper. 3*				
1	197	$\frac{1}{4}$	2	3'10"
1a	197	$\frac{1}{4}$	"	Slight
2	190	$\frac{1}{2}$	"	Slight
4a	191	$\frac{1}{2}$	"	Very slight
Exper. 4*				
1	188	$\frac{1}{4}$	2	Severe
1a	188	$\frac{1}{4}$	"	2'30"
2	185	$\frac{1}{2}$	"	Good
2a	186	$\frac{1}{2}$	"	Moderate

* Tests made in duplicate. Mixtures for Expers. 1 and 2 were kept at 45 C.; those for Expers. 3 and 4 were kept at 38 C.

The interpretation of these results is of fundamental importance. It may be assumed that the heat of inactivation destroys the ferment, but it is more likely that it causes a change in the matrix. The indication is that the matrix, probably but a small part of the total protein of the serum, like fibrinogen is very labile. It may be imagined that

the action of heat consists first in a dispersion followed by an intramolecular change analogous to that which occurs in heat coagulation. Once denatured by heat, the capacity of the matrix to produce poison is lost.

Toxicity of the Agar Deposit.—Rat serum on incubation with agar yields a precipitate which is more abundant than that obtained with guinea-pig or rabbit serum, and on centrifugation this precipitate is thrown down along with the agar. Since an adsorbing surface such as that presented by agar might possibly take up anaphylatoxin from the serum, many efforts were made to detoxify a serum by repeated treatments with agar gel. The results seemed to indicate that the poison could be removed in this way.

If an adsorption of some of the poison does take place, then it should be possible to obtain indications of its presence by digestion of the agar precipitate with distilled water. Several attempts were made with this object in view.

In one experiment a sol-gel mixture, consisting of 2.5 c.c. of sol and 10 c.c. of rat serum, after icing for 1 hour, was incubated at 37 C. for 20 minutes and then centrifugated. The supernatant serum in dose of 0.25 c.c. gave a good shock when tested at once and after 10 minutes. The agar deposit was rubbed up with 10 c.c. of distilled water and placed at 45 C. for 1 hour, after which it was centrifugated for 5 minutes and the clear aqueous extract injected. The first test with 5 c.c. caused typical acute death in 2 minutes 40 seconds in a guinea-pig weighing 206 gm.; and a second test with 2.5 c.c. was fatal in 30 minutes to a guinea-pig weighing 170 gm.

In another experiment a similar result was obtained. The sol-gel mixture in this case consisted of 2.2 c.c. of sol and 22 c.c. of serum; after being iced for 1 hour it was placed at 38 C. for 15 minutes and then centrifugated. The supernatant serum was fatal in dose of 0.25 c.c. The agar deposit from this serum was taken up with 22 c.c. of distilled water and digested for 1 hour at 38 C., after which the mixture was centrifugated and the aqueous extract tested. Injected in dose of 5 c.c. into a guinea-pig weighing 200 gm. it caused typical shock and death in 3 minutes 10 seconds.

The question arose as to whether the toxicity of the extract was due to the re-solution of an adsorbed or occluded poison, or whether it was to be correlated with the toxicity of organ extracts. The former view was supported by the following experiment in which the serum precipitate was excluded.

A clear toxic rat serum, the lethal dose of which was 0.25 c.c., was mixed with an equal volume of gel (3.75 c.c.) and set aside at 0 C. for 21 hours. The mixture was then centrifugated; 1 c.c. of the clear serum when injected into a guinea-pig produced but slight effect. Evidently, the toxicity had greatly decreased, possibly as the result of adsorption. The agar deposit was then

digested with 15 c.c. of salt solution at 50 C. for 45 minutes and centrifugated. The injection of 10 c.c. of this extract into a guinea-pig weighing 230 gm. caused death in 2 minutes 40 seconds, while 5 c.c. produced but slight effect. The agar extracted a second time with 15 c.c. of salt solution, at 50 C. for 1 hour, gave an extract which in dose of 10 c.c. had practically no effect.

It is evident, therefore, that the agar may adsorb or what is more likely, occlude a certain amount of the serum constituents including the poison. This fact serves to explain why, at times, apparently large doses of agar fail to toxify a serum.

SUMMARY

Rat serum can be toxified with agar in $7\frac{1}{2}$ minutes so that 0.25 c.c. will cause acute fatal shock; after incubation for only $2\frac{1}{2}$ minutes, 1 c.c. may be fatal. The reaction concerned in the production of anaphylatoxin is one of great speed. Agar and trypanosomes can work at the same speed since they can toxify 1 c.c. in $2\frac{1}{2}$ minutes.

The individual sera show some variation in the ease with which they can be toxified. This is not due to the age of the serum or to the presence of lipoids. Normal rat serum, without any addition, on long incubation may become toxic so that 1 c.c. will be fatal.

The physical state of the agar is an important factor in the production of the poison. Its inducing power is not affected by sterilization at 140 C.

The sol at 37 C. is inferior to the solid or semisolid agar, and the best results are obtainable by the sol-gel method.

A mixture (No. 1) containing 0.0005 c.c. of agar per cubic centimeter of serum can be toxified so that 1 c.c. will be fatal. This amount of agar solution contains 0.0025 mg. of dry agar—a quantity so small as to exclude it as the source of the poison, and a *reductio ad absurdum* of Friedberger's theory of anaphylatoxin-production. The ratio of dry agar to serum in this mixture is 1:400,000.

The most convenient mixture, probably, is one containing 0.1 to 0.05 c.c. agar per cubic centimeter of serum. The reaction is not inhibited in a mixture consisting of equal parts of agar and serum.

Some incubation of the agar serum at or near 37 C. is necessary to the production of the poison. Incubation at 45 C. yields about as active a poison as at 37 C.

The poison is not destroyed when kept at 56 C. for 24 hours; it is partially destroyed in half an hour at 70 C., and apparently completely in 5 minutes at 100 C.

The apparent variation in the toxicity of a given treated serum is due to the varying resistance of the recipients.

Attempts at demonstrating the presence of a causal ferment, by progressive dilution, gave results which are shown to be due to the residual agar. No positive evidence that agar adsorbed a ferment could be established.

Serum which has been heated to 56 C., or even to 60 C., can be toxified by a treated serum, or by the agar deposit, or even by plain agar, but not as readily as unheated normal serum. The serum is weakened by heating to 50 C., but not by heating to 45 C. The facts indicate that the matrix of the poison is very labile.

The agar deposit may hold, probably by occlusion, one or more fatal doses of the poison.

V. EFFECT OF MULTIPLE DOSES OF ANAPHYLATOXIN

F. G. NOVY AND P. H. DEKRUIF

SYNOPSIS

INTRODUCTION

METHODS

INJECTION OF GUINEA-PIGS WITH FROM 1 TO 40 LETHAL DOSES

EFFECT ON TIME OF DEATH; ON COAGULATION

THE IN-VITRO ANTICOAGULANT ACTION

INJECTION OF WHITE RATS WITH FROM 1 TO 75 GUINEA-PIG

LETHAL DOSES

NATURAL IMMUNITY; RAPID DISAPPEARANCE FROM THE BLOOD

HETEROLOGOUS ANAPHYLATOXIN

INJECTION OF RABBITS WITH FROM 20 TO 40 GUINEA-PIG LETHAL

DOSES

NATURAL IMMUNITY

TOXICITY OF ANTISHEEP RABBIT SERUM

SUMMARY

It was manifestly impossible to study the effects of multiple doses as long as the anaphylatoxin possessed a relatively feeble action. The lethal dose of the toxic serum, as prepared by previous workers, was usually about 3 c.c. per 200 gm. of guinea-pig. Doerr¹ stated that the smallest lethal dose known of guinea-pig anaphylatoxin was 2 c.c., tho 1.5 c.c. had been observed to kill in 1 hour. It has been shown in the work reported here that it is possible, with agar, to render guinea-pig serum toxic so that it is acutely fatal in dose of less than 1 c.c. Notable exceptions to Doerr's statement were made by Jobling and Petersen² and by Bronfenbrenner,³ who were able, respectively, to toxify guinea-pig serum so that it killed in dose of 0.31 and 0.5 c.c. The development of a speedy method whereby it was possible to prepare a toxic rat serum the lethal dose of which was but 0.2 to 0.25 c.c., opened the subject to investigation.

It seemed essential to the understanding of the problem of specific anaphylaxis that the behavior of anaphylatoxin be studied under the conditions mentioned. The mystery of the acute specific shock was largely due to the lack of definite facts regarding the amount and nature of the poison concerned. Occasional recoveries, when large amounts of antigen were given at the second injection, would at times

¹ Kolle and Wassermann's Handb. d. pathogen. Mikroorganismen, 1913, 2, p. 1105.

² Jour. Exper. Med., 1914, 19, p. 485.

³ Ibid., 21, p. 480.

be interpreted as due to excessive poison-production, a conclusion implying that a large amount of poison was less toxic than a small amount. It was therefore of interest to ascertain whether there was any difference between the toxic effects when one and when a number of lethal doses were administered. Incidentally, it was hoped that some light would be thrown upon the relation of the dose of anaphylatoxin to the noncoagulability of blood.

The experiments which were made in this direction led to some striking results, not only as regards coagulation, but also as to the effect of large doses in guinea-pigs and especially in rats. The information thus acquired became of extreme value in attacking the problem of anaphylaxis.

The rat anaphylatoxin was prepared by the sol-gel method, using Mixture 6, which yields maximal toxicity in the shortest possible time. After incubating the iced mixture for 15 minutes, a small portion was removed, centrifuged, and tested; if acute death followed the injection of 0.25 c.c. of the serum, the entire mixture was at once centrifuged and the serum used for the experiment. Usually, incubation for 15 minutes gave the desired lethal dose, but if such was not the case, the mixture was retested after a further incubation of 15 minutes. In order to avoid any decrease in toxicity through possible reversion changes the sera were used as soon as prepared. There is no reason to believe, however, that the anaphylatoxin varies appreciably when kept for a short time at room temperature.

The injection, when small doses were administered, took not over 20 seconds. The difference in time between the end of such injection and the last nasal twitch was taken as the time necessary to kill. In several instances, the injections were made slowly but steadily, extending over 2 to 4 minutes, and in such cases the time necessary to kill is expressed in 2 figures; the first of these represents the lapse of time from the end of injection, the second from the start to the final nasal twitch. The speed of the injection, it will be shown, has a distinct influence on the coagulability of the blood.

INJECTION OF GUINEA-PIGS

The results of these experiments are summarized in Table 57. It should be stated that for these tests, 9 different toxic pools were employed, all possessed of an initial lethal dose of 0.25 c.c. It is indeed possible that some may have killed in dose of 0.2 c.c. One pool served for Tests 1, 2, 4, 5, and 13; another for 3, 12, and 14; another for 8 and 15; another for 9 and 16, while separate pools were used for each of the remaining tests. The injections, as usual, were intravenous.

It is seen in Table 57 that tho the number of lethal doses ranged from 1 to 40, the death point was essentially the same in all. In other words, the symptom complex of acute anaphylactic asphyxia requires a

fairly constant time regardless of the number of toxic doses employed. The type of the shock, however, varies distinctly with the number of lethal doses. With moderate amounts, the usual typical symptoms of oncoming dyspnea, spasms, and violent convulsions are noted; with the larger doses the overwhelming effect is such that the convulsions and even spasms are missed, the picture being that of an intense quiet shock. In such case, the animal when released lies limp, with respiration all but suppressed, and shows at most a few tremors of the feet. Incidentally, it may be said that this type of shock is often met with when injecting large amounts of normal sera, cyanid, peptone, etc.

TABLE 57
EFFECT OF MULTIPLE LETHAL DOSES OF ANAPHYLATOXIC SERUM ON GUINEA-PIGS

Guinea-Pig		Serum		Result		Clotting Time in Test Tube (min.)
No.	Weight	c.c. (Intravenously)	Lethal Doses			
1	177	0.25	1	3'40"	Typical shock	5
2	182	1.25	5	3'15"	" "	11
3	190	1.25	5	3' 5"	" "	6
4	190	2.5	10	3'40"	" "	8
5	205	"	"	3'50"	" "	11
6	188	"	"	3' 5"	" "	
7	192	"	"	3'	" "	
8	208	"	"	3'15"	Quiet shock	8
9	215	"	"	2'40"-5'	" "	11
10	192	5.0	20	3'	Typical shock	
11	205	"	"	4'20"	Quiet shock	0
12	175	"	"	2'55"	" "	
13	210	10.0	40	3'50"-4'25"		25
14	213	"	"	3'55"-4'25"		17
15	205	"	"	1'55"-4'		⊕
16	206	"	"	0'15"-4'15"		⊕
17	200	"	"	1'10"-4'25"		⊕

⊕ Incoagulable.

In all cases, the autopsy, made from 3 to 4 minutes after death, revealed the same typical picture: maximal distention of the lungs and, usually, in animals which had received 10 or more lethal doses, enormous petechiæ in the lungs; heart beating and blood perfectly fluid without sign of clot. The absence of clot after injection of the largest doses of this serum, is all the more remarkable since large doses of rabbit anaphylatoxin (6 c.c.) almost invariably produce rapid clotting.

Effect on Coagulation.—In all but 4 of the animals, an in-vitro test of the coagulability of the blood was made. For this purpose, before cutting open the heart, about 0.5 c.c. of the blood was withdrawn by means of a syringe and transferred to a small test tube (8 to 9 mm. in diameter); observations were then made every minute thereafter. The time needed to form a solid clot (++++) is indicated in the table.

The transfer to the glass considerably accelerated the clotting process, the blood in the heart being often found perfectly fluid for some minutes after the coagulation had taken place in the test tube.⁴

The coagulation time, as given in the table, refers to the time which elapsed after withdrawal of the blood from the heart, this being done 3 minutes after death. When several transfers of blood were made from the heart, for example at 3, 4, and 5 minutes after death, these portions gave a varying coagulation time; that for the second portion was less while that for the third was nearly the same as for the first. More extended observations are desirable, but those made are suggestive of the 'courbe oscillante' of DeWaele.⁵

Of special interest are the tests in which 10 c.c. of serum were injected. In Test 14, in which the injection took 30 seconds, the blood coagulated in the test tube in 17 minutes; in Test 13 an almost equally rapid injection gave a blood which formed a full, but very soft, clot in 25 minutes. On the other hand, in the last three of the series, the same amount of serum being administered but very slowly, the blood was almost incoagulable. This state is indicated in the table by the sign \ominus . In Tests 15 and 16, a very minute clot (+), about 2 mm. in diameter, which could have been easily overlooked, was detected in 4 and 10 minutes, respectively, but the process went no further. In No. 17 the clot became a trifle larger in 12 minutes (++), but this also was arrested; the serum which had been injected into this guinea-pig had been heated to 50 C. for half an hour with the expectation of weakening its thrombin content and thus securing a minimal clot. The heating process evidently had the opposite effect. With the appearance of the minute clot mentioned the blood seemed to increase in viscosity, but this condition soon passed away, the blood becoming as fluid as before.

In Test 11, in which the injection time was short (not recorded), there was no sign of coagulation for 3 hours; the observation was then discontinued until the following morning, when a solid coagulum was found. In No. 9, the injection was given in divided doses, 0.5 c.c. every half minute, but this did not have any perceptible effect either on the time of death or on the coagulation. With the smaller doses (Nos. 1 to 9) the coagulation was slightly retarded, requiring about 2 to 4 times as long as normal blood.

⁴ Delayed coagulation of the blood in asphyxia is a well-known fact. Friedberger (Ztschr. f. Immunitätsf., 1910, 8, p. 263) found that the blood of guinea-pigs, killed by blow or compression, did not coagulate in 15 minutes; that obtained after death from injection of anaphylatoxin also remained free from clot for from 10 to 20 minutes. These figures presumably refer to the intracardial condition.

⁵ Ztschr. f. Immunitätsf., 1913, 17, p. 314.

This method of testing the coagulability of the blood is capable of improvement, especially in view of the fact that the withdrawal of blood from the heart through a needle, introduces a factor of contact which can be avoided. The heart pipet method, which was eventually time of death or on the coagulation. With the smaller doses (Nos. 1 (Parts VII and IX), might be expected to give a strictly noncoagulable blood, were it applied to guinea-pigs injected with large multiple doses of anaphylatoxin.

The conclusion to be deduced from these tests is that rat anaphylatoxin when injected tends to retard either directly or indirectly the coagulation process. The very slow injection of a large dose may give a practically noncoagulable blood.

The In-Vitro Anticoagulant Action of Anaphylatoxin.—As shown in Table 57, the blood of guinea-pigs injected with anaphylatoxin, when transferred to a test tube, shows some retardation of coagulation. If this was due to the mere presence of the poison, it should be possible to secure a similar retardation, if not complete incoagulability, by making an in-vitro mixture of anaphylatoxic serum and fresh normal blood, and the results of a large number of experiments made with this object in view certainly indicated a marked inhibitory action.

One method of procedure was to place 0.25 c.c. of the rat anaphylatoxin in a small test tube and then to add 1 or 2 volumes of blood, drawn as rapidly as possible from the heart. The two were mixed and the fluid was then examined every minute until solid coagulation took place. A better method seemed to be to draw up the blood, either from the exposed heart or from the jugular vein, into a syringe containing anaphylatoxin. By pulling back the piston, the two fluids were quickly mixed, and the mixture was then at once discharged into a test tube. In these tests care was taken to employ an anaphylatoxin which was fatal in dose of 0.25 c.c.

Normal guinea-pig blood, drawn from the heart, clots in 4 minutes or less, but if a series of punctures is made on the same heart, through the pleural wall, extreme variations in coagulability will be encountered. Thus, in a series of 10 consecutive punctures, the range was from 1 minute to incoagulability. This fact made it plain that satisfactory results could be obtained only when the blood was drawn from an exposed heart or vein.

In a test according to the latter method, 0.25 c.c. of jugular blood plus 0.5 c.c. of anaphylatoxin coagulated in 14 minutes, while a mixture of equal volumes clotted in 7 minutes. This test was repeated a number of times with essentially the same result. Rabbit blood seemed to

be retarded by rat anaphylatoxin to a less extent than guinea-pig blood, while rat blood was apparently still less delayed.

There is no doubt but that the rapid admixture of anaphylatoxin with normal blood considerably delays coagulation; the larger the amount of the former the more marked is this result. Control tests, in which the blood was drawn into a syringe containing 1 or 2 volumes of normal rat serum, invariably coagulated in about 1 minute.

INJECTION OF WHITE RATS

The behavior of multiple doses of anaphylatoxin in the guinea-pig having been studied, it was next in order to extend the observations to the white rat, which apparently had never been used for this purpose. It may be stated, however, that Ritz and Sachs⁶ found that white mice were not killed by anaphylatoxin, but were killed by peptone injections. Since the mice had died of specific anaphylactic shock, it was inferred that more poison was produced in vivo than had been injected, or else that the mouse-serum anaphylatoxin differed from that of the guinea-pig. It was therefore an interesting question as to what would be the behavior of the rat in response to one or more doses of the homologous anaphylatoxin. The tests made with that object in view revealed a remarkable example of natural immunity or tolerance of the poison.

The rat anaphylatoxin was prepared in the same way as that used for the guinea-pigs. Five different pools were used. One of these with a lethal dose of 0.25 c.c. was employed for the first 6 tests given in Table 58; another pool of the same strength was used for Tests 9 and 10. The lethal dose of the serum used for Test 11 was 0.2 c.c. That for No. 7 was a trifle weak, 0.25 c.c. giving a near-kill; the number of lethal doses injected into No. 7 was therefore between 10 and 20. The serum used for No. 8 also failed to kill in 0.25-c.c. dose and hence the number of lethal doses was between 20 and 40. The failures to kill in these titrations may be simply indicative of individual resistance.

The injections were all intravenous; at first the caudal vein was used, but it was soon found that the femoral vein was more convenient. The injection time of the first 6 tests was short, probably from 5 to 20 seconds according to the dose. With the larger dose (Nos. 8 to 11) the time was respectively $\frac{3}{4}$, $1\frac{3}{4}$, $1\frac{1}{2}$, and 1 minute.

Even a cursory examination of Table 58 shows that the rat possesses an extraordinary tolerance of the homologous anaphylatoxin. The injection of 20 guinea-pig lethal doses had practically no effect. With 40 of such lethal doses, some respiratory disturbance and slight

⁶ Centralbl. f. Bakteriöl., R., 1912, 50, Beiheft, p. 45; i. e., Bericht üb. d. 5te Tagung d. Fr. Ver. f. Mikrobiöl. in Dresden, 1911.

transitory jerks were noted. With 75 doses the result was more severe but recovery was prompt.

It is to be noted that Rat 11, which received 75 guinea-pig lethal doses, actually was injected with a volume of serum corresponding to 10% of its body weight. A normal serum if given to a guinea-pig in dose representing 5% of its body weight will almost invariably kill. In this case an intensely toxic serum was given in twice that amount without causing death. After the injection of 15 c.c. of the toxic serum the rat was at first slightly excited, then became prone and respiration gradually faded to nil; after about half a minute it slowly returned, and within 2 minutes the rat had apparently recovered. By contrast, Rat 9, which received 10 c.c. of the serum (75 per kilo), showed slight jerks while being injected but none thereafter; the respiration was slightly increased and the animal was quiet.

The anaphylatoxin injected into Rat 11 was fatal to a guinea-pig of 247 gm. in dose of 0.25 c.c. in 3 minutes 20 seconds; that is to say, it was toxic in 1 c.c. per kilo of guinea-pig. The dose of 15 c.c. given to the rat represents 100 c.c. per kilo of rat. Weight for weight, the rat can tolerate more than 100 times as much anaphylatoxin as the guinea-pig. This fact is all the more remarkable when it is realized that the rat serum, in a given time, can yield an anaphylatoxin which is easily 10 times more toxic than that of the guinea-pig. If it is assumed that the plasma is equally labile, it will be seen that the rat would be extremely susceptible to shock were it not for some natural protection. The tolerance noted is therefore suggestively teleologic. It will be shown that the rabbit likewise shows a tolerance for anaphylatoxin.

TABLE 58
EFFECT OF MULTIPLE GUINEA-PIG LETHAL DOSES OF HOMOLOGOUS ANAPHYLATOXIC SERUM ON
WHITE RATS

Rat		Serum		Result
No.	Weight	c.c. (intravenously)	Doses	
1	110	0.25	1	Nil
2	115	0.5	2	"
3	119	1.25	5	"
4	110	1.25	5	"
5	135	2.0	8	Increased respiration
6	145	2.5	10	Nil
7	120	5.0	10-20	"
8	125	10	20-40	"
9	132	10	40	Slight
10	155	10	40	"
11	151	15	75	Severe
12	158	15 (g.p.s.)	7.5-10	Increased respiration

Several explanations of this tolerance can be readily formulated. For instance, the resistance might be assumed to reside in the cells of the body, the cytoplasm offering extreme resistance to dislocation; or, it could be imagined that the plasma of the blood and of the cells exerted a destructive action which could be conceived as oxidative, lytic, or reversible. The action of the blood on the poison is one that is open to experimentation. By means of the transfusion method it should be possible to find out how long the injected poison remains in the circulation. Unfortunately, but one experiment of this kind was made; still it suffices to show that the poison disappears from the circulation of the rat in less than 15 minutes.

Rat 11, which had received 75 guinea-pig lethal doses, was selected for the transfusion experiment, the method of work being the same as that used in connection with the experiments recorded in Table 65, Part VI. After the rat had apparently completely recovered from the effects of the injection, 2 c.c. of blood were drawn from the heart and injected intravenously into a guinea-pig of 178 gm. The result was nil; there was no sign of respiratory disturbance or spasm, and at most a very slight peripheral irritation. The total time which elapsed from the end of the injection into the rat to the end of the injection into the guinea-pig was 15 minutes, while the transfer time from the moment the blood entered the syringe until it was injected into the recipient was 25 seconds. Hence, it follows that in the 'reaction time' of 14 minutes 35 seconds, the time within the body of the rat, much of the poison had disappeared.

On the supposition that the rat contained 10% of its body weight of blood (15 c.c.), and that the volume of the blood after the injection of 15 c.c. of serum had not decreased, it follows that 30 c.c. of the mixed blood represented 75 lethal doses of poison. Consequently, the 2 c.c. transfused, should contain 5 lethal doses; the results show that it actually had little or none. This fact certainly indicates a rapid removal of the poison from the circulation of the rat. That the blood of this animal was not quite normal is seen in this, that immediately after the transfusion, 0.5 c.c. of blood drawn from the heart and transfused to a small test tube began to clot in 4 minutes and became solid in 5. Ordinarily, rat blood will coagulate in 1 or 2 minutes.

Heterologous Anaphylatoxin.—In the first 11 tests given in Table 58, the rats were injected with the homologous or rat anaphylatoxin. It was possible that the rat could change or destroy the homologous

poison more readily than the heterologous; the latter might in that case be poisonous for the rat. To test this point, an anaphylatoxin was prepared by the sol-gel method with guinea-pig serum. A preliminary test showed that 2 c.c. were fatal to a guinea-pig of 200 gm. in 3 minutes 15 seconds, and that 1.5 c.c. gave a near-kill. Fifteen cubic centimeters of this anaphylatoxin, representing therefore from 7.5 to 10 guinea-pig lethal doses, when injected into Rat 12 had no effect. Clearly, the tolerance of the rat holds for the heterologous as well as for the homologous poison.

INJECTION OF RABBITS

Friedberger and Castelli⁷ tested the effect of antiserum from immune rabbits on normal rabbits and found that the latter showed a distinct tolerance for the poison which was present in such serum. Of a serum which was toxic in dose of 0.5 c.c. per 200 gm. of guinea-pig, 9 c.c. were injected into a rabbit of 770 gm. without producing any effect; this represents 18 guinea-pig lethal doses, or 4.7 per 200 gm. of rabbit. In another test a rabbit of 1600 gm. received 10 c.c. of a serum which was fatal in dose of 0.16 c.c. per 200 gm. of guinea-pig; this represents 62.5 guinea-pig lethal doses, or 7.8 per 200 gm. of rabbit.

Doerr and Weinfurter⁸ obtained a similar result. In the one test which they made with an antiserum they gave a rabbit of 800 gm. 5 c.c. of a serum which was fatal in dose of 0.1 c.c. per 200 gm. of guinea-pig; this corresponds to 50 guinea-pig lethal doses, or to 12.5 per 200 gm. of rabbit. It will be seen from these tests that weight for weight the rabbit can tolerate more than 12 guinea-pig lethal doses.

Inasmuch as the toxicity of the antiserum must be ascribed to the anaphylatoxin which is made in vivo in the rabbit, it is evident that the rabbit must possess a marked tolerance for the poison. In view of this fact it was to be expected that an injection of an anaphylatoxin prepared in vitro by the action of agar on rat serum would be equally tolerated. The experiment confirmed this reasoning.

A toxic rat serum, having a lethal dose of 0.25 c.c., was injected intravenously into 2 young rabbits. Rabbit 1 of 585 gm. was given 5 c.c., and Rabbit 2 of 595 gm. 10 c.c. of this agar anaphylatoxin without the slightest effect being produced in either case. The dose per kilo was therefore 8.5 and 16.8 c.c. respectively, while 1.25 c.c. was the

⁷ Ztschr. f. Immunitätsf., 1910, 6, p. 267.

⁸ Centralbl. f. Bakteriöl., I, O., 1912, 63, p. 420.

lethal dose per kilo of guinea-pig. Weight for weight, therefore, the rabbits tolerated from 6.8 to 13.4 times as much poison as would kill a guinea-pig.

Instead of 13.4 guinea-pig lethal doses per 200 gm. of rabbit, it would be of interest to test 50 or more doses in order to ascertain the full extent of the tolerance possessed by the rabbit. The blood and serum of rabbits treated with sheep corpuscles can become very toxic as is well known. The amount of anaphylatoxin present in such treated animals may be large as will be seen from the following observation. A rabbit of 1720 gm. gave a serum which caused acute fatal shock in 200-gm. guinea-pigs in dose of 0.05 c.c. If it is assumed that 10% of the body weight is blood, this would give 172 c.c., representing 103 c.c. of serum. This amount of serum would mean that the rabbit carried 2060 guinea-pig lethal doses, implying an extraordinary resistance to the poison; that is, 240 guinea-pig lethal doses per 200 gm. of rabbit. Doerr and Weinfurter estimated that 2 of their highly toxic rabbits carried from 1700 to 2500 guinea-pig lethal doses. It will be shown in Part VI that, at times, the normal rabbit may carry in its blood 100 of such fatal doses; that is, 10 per 200 gm. rabbit.

It will be seen that the rabbit like the rat is resistant to the poison. Their respective sera, however, as has been shown, differ greatly in the ease with which they are toxified.

SUMMARY

Rat anaphylatoxin, in amounts representing from 1 to 40 lethal doses, invariably caused death in guinea-pigs, the time being essentially the same.

The larger doses gave an intense quiet shock; the autopsy findings in all cases were typical, but with larger doses the petechiæ in the lungs were most pronounced; the blood was always free from clot.

The blood when transferred to a test tube showed delay in coagulation. Slow injection of very large doses gave an almost incoagulable blood.

The in-vitro mixtures of rat anaphylatoxin and normal blood likewise showed retarded coagulation.

The white rat possesses a remarkable immunity against anaphylatoxin; weight for weight it can tolerate more than 100 times as much as the guinea-pig. The dosage with serum reached 10% of the body weight.

A transfusion experiment indicated a rapid disappearance of the anaphylatoxin from the blood.

The heterologous anaphylatoxin, prepared with guinea-pig serum, was tolerated the same as the homologous.

The rabbit like the rat is not affected by large doses of anaphylatoxin and this fact parallels the behavior of rabbits on injection of large doses of the toxic antisheep immune rabbit serum. It is this tolerance of the rabbit for the poison that permits the formation of large amounts of anaphylatoxin during life. Antisheep guinea-pig serum is not toxic since the formation of anaphylatoxin to the extent of a single lethal dose would mean the death of the animal. This tolerance likewise accounts for the known resistance of the rabbit to specific anaphylaxis.

VI. EFFECT OF INTRAVENOUS INJECTIONS OF AGAR

F. G. NOVY AND P. H. DEKRUIF

SYNOPSIS

INTRODUCTION

INJECTION OF GUINEA-PIGS

EFFECT OF AGAR SOL AT 50 C.; AT 37 C.

ATYPICAL RESULTS WITH GEL SUSPENSIONS

ACTION OF SOL-GEL IN SALT SOLUTION; IN DISTILLED WATER

SIGNIFICANCE OF ATYPICAL SHOCK; OF AGAR INTOXICATION

LETHAL DOSE ABOUT 10 MG. PER KILO

INJECTION OF RABBITS

EFFECT OF DILUTE SOL-GEL

INJECTION OF RATS

EFFECT OF DILUTE SOL-GEL

TRANSFUSION OF BLOOD OF RATS SHOCKED WITH AGAR

TRANSFUSION OF BLOOD OF NORMAL RATS

TRANSFUSION OF BLOOD OF GUINEA-PIGS SHOCKED WITH AGAR

TRANSFUSION OF BLOOD OF RABBITS SHOCKED WITH AGAR;

PRESENCE OF ANAPHYLATOXIN IN THE BLOOD OF NORMAL

RABBITS

AGAR AND ENDOTOXIN

SUMMARY

In view of the fact that agar, when added to a serum, readily produces anaphylatoxin, it seemed rational to expect that a like result could be obtained in vivo. In other words, it should be possible to produce a typical anaphylatoxic poisoning, that is, the anaphylactic shock, by an intravenous injection of an agar solution or suspension. The early tests made with this object in view were far from encouraging, for the reason that they were made at a time when the optimal conditions for the in-vitro production of agar anaphylatoxin were still unknown. In the end the desired result was attained; not only was it possible to secure perfect anaphylactic shock in guinea-pigs, but also corresponding reactions in rabbits and rats. Furthermore, and as an offset to the possible objection that such shocks were due to mere agar embolism, it was shown by transfusion experiments that a typical anaphylatoxin was formed in the blood of the injected rats and guinea-pigs, and possibly in that of similarly treated rabbits.

INJECTION OF GUINEA-PIGS

Agar Sol.—In one series of experiments the agar was injected as a sol at a temperature of 50 C. This temperature was employed with the idea in mind that the more perfect the state of dispersion, the

better would be the result. Previous in-vitro tests had shown that the 50 C. sol was capable of producing naphylatoxin. For these tests, the sterile 0.5% agar was liquefied by heating in the water bath at 100 C. for about 15 minutes, after which it was placed in a Roux bath at 50 C. for a like time. The hydrosol was then injected, intravenously, in varying amounts, as indicated in Table 59, which also gives the speed of the injection.

TABLE 59
INJECTION OF 0.5% AGAR HYDROSOL (50 C.) INTO GUINEA-PIGS

Guinea-Pig		Agar Hydrosol		Result
No.	Weight	c.c. (intravenously)	Injection Time (sec.)	
1	210	1.0	20	Nil
2	215	1.5	28	Few jerky spasms
3	183	2.0	25	Increased respiration
4	207	3.0	35	Increased respiration and few slight jerks
5	210	5.0	40	Nil
6	240	7.5	45	Increased respiration, slight peripheral irritation
7	215	10.0*	65	6'20". Typical shock and autopsy

* This represents 232 mg. of dry agar per kilo of body-weight.

It will be seen from the table that of 7 guinea-pigs which received varying amounts of sol ranging from 1 to 10 c.c. only the one receiving the latter amount died. In this animal (No. 7) the shock was perfectly typical, with dyspnea, spasms, and convulsions; the autopsy was likewise, showing maximal distention of lungs, heart beating, blood fluid, and no clot.

Since the death of Guinea-pig 7 was so clearly one of typical anaphylactic shock, confirmatory results were confidently expected. But a new agar solution, prepared and tested on the following day, gave most disappointing results. Of 6 guinea-pigs which received an intravenous injection of 10 c.c. of the 50 C. sol, 5 showed no other effect than slightly increased respiration; one died in 1 minute 50 seconds, in an atypical manner supposedly due to the rapid injection (35 seconds) of the hot sol. The single typical result was therefore rather exceptional and was probably dependent on some slight condition easily overlooked.

It is worth noting that frequently 10 c.c. of agar sol, representing 50 mg. of agar (200-250 mg. per kilo), can be given without any ill effect. It will be shown that with the agar in the proper state even as little as 9.4 mg. per kilo of body weight may be fatal.

The injection of hydrosol at 37 C. (obtained by placing the liquefied agar in the Roux bath for several hours) likewise gave little or no

result. At most a few slight jerks would follow the injection of from 5 to 10 c.c. of such sol.

Agar Gel.—Other attempts were made to produce shock by injection of suspensions of solid gel in 0.85% salt solution. These were made when experience had shown that the gel was more reliable than the sol as a producer of anaphylatoxin. The previously liquefied agar was first placed in cracked ice to gel, and the solid mass on shaking readily changed into a semifluid state. When 1 part of this semigel was shaken with 4 parts of salt solution, it yielded a fine suspension which could be readily injected intravenously. Such suspension, when slowly injected in 2-c.c. amount into a 230-gm. guinea-pig, produced marked peripheral irritation, defecation, and slight jerky spasms, besides a drop in temperature to 36 C. Three cubic centimeters of the same suspension produced death in a 230-gm. guinea-pig in 1 hour; while a like amount in another animal of 190 gm. caused death in 2 minutes. The symptoms and findings were not those of typical anaphylaxis (lungs collapsed, clot in heart).

On another occasion tests were made with a like suspension which had been vigorously shaken for 3 minutes and then iced for 15 minutes. As much as 7.5 c.c. of this were injected into a 270-gm. guinea-pig without producing death, tho the shock was severe and recovery slow. The same dose of another suspension, made on the same day, killed in 3 minutes, but here, as before, collapse of lungs and heart clots showed that the object was not attained. Injections of 3 and 5 c.c. of this suspension resulted at most in a few slight jerky spasms and a slight dyspnea.

Agar Sol-Gel.—It was evident from the inconstant results aforementioned, that a perfectly homogeneous suspension of the gel could not be obtained by mere shaking of the semifluid mass with salt solution, and that as long as gross particles of agar were present, an atypical shock would be produced. Accordingly, what seemed to be a more promising method was tried.

The agar after being liquefied by heating at 100 C. for 15 minutes was placed in a Roux bath at 37 C. for 2 hours to form a hydrosol. This was then added to one or more parts of salt solution, also at 37 C. and thoroughly shaken for 5 minutes, after which the resulting fluid was placed in cracked ice for 1 hour. It remained perfectly clear with no sign of agar clumps. In this liquid the agar is therefore in an extreme state of dispersion or division and what is very important, these particles are presumably in the gel form. The results (Table 60) obtained with this sol-gel met all expectations.

TABLE 60
INJECTION OF AGAR SOL-GEL (0 C.) INTO GUINEA-PIGS
(THE SOL AND SALT SOLUTION, BOTH AT 37 C., WERE MIXED AND ICED FOR 1 HOUR)

Mix- ture	Guinea-Pig		Agar Sol-Gel			Result
	No.	Weight	c.c. (intraven- ously)	Agar* (mg.)	Injection Time (sec.)	
1:1	1	273	1.5	3.75	20	Slight. In $\frac{3}{4}$ minute slight jerky spasms, some dyspnea
	2	250	3.0	7.5	40	Very slight. In 6 minutes mild peripheral irritation
	3	300	5.0	12.5	60	Slight. In 1 minute few slight jerky spasms, respiration becoming rapid and shallow and later very labored
	4	325	5.0	12.5	30	7. Atypical shock; at once on side, limp, very shallow respiration. Autopsy showed lungs partially distended, heart not beating, clot present
1:4	5	320	4.0	4.0	60	430". Typical shock; no effect for 2 minutes, then peripheral irritation, dyspnea, spasms, thrown. Autopsy, 5 minutes after death, showed maximal distention of lungs, hemorrhages; heart stopped, blood fluid, no clot
	6	318	4.0	4.0	50	Severe shock. No effect for 2 minutes, then peripheral irritation, severe dyspnea, thrown in 6 minutes, but up 4 minutes later. Recovered
	7	315	5.0	5.0	50	6. Typical shock; no effect for 2 minutes, then peripheral irritation, moderate dyspnea, spasms, thrown. Autopsy 5 minutes after death; same findings as in No. 5
	8	325	5.0	5.0	30	5. Typical shock; peripheral irritation in $\frac{1}{2}$ minute, then dyspnea, spasms, convulsions, thrown. Autopsy 10 minutes after death showed same findings as in No. 5. Very slight apex beat
	9	315	10.0	10.0	60	Very slight. Mild peripheral irritation, in 3 minutes slight dyspnea, no spasms
1:9	10	275	5.0	2.5	30	Slight. Mild peripheral irritation, few jerky spasms, labored respiration
	11	275	10.0	5.0	60	Severe. Very excited, peripheral irritation, marked dyspnea, spasms, jumps, not thrown, in 5 minutes quiet but depressed

* In the fatal cases, Nos. 4, 5, 7, and 8, the amount of agar per kilo of body weight was 38, 12.5, 16, and 15 mg., respectively.

It will be recalled from the previous work that the in-vitro sol-gel mixtures yield the most rapid production of anaphylatoxin; in this case essentially the same conditions were supplied in vivo. That a certain state of the agar, rather than quantity, is necessary to the production of a severe shock will be seen on comparison of Tables 59 and 60. Thus, 10 c.c. of the undiluted sol, at 37 C. or at 50 C., containing 50 mg. of agar, repeatedly produced little or no effect, tho in one instance (Table 59, No. 7) it did cause typical shock and death.

By contrast, in the fatal tests recorded in Table 60 (Nos. 5, 7, and 8) the amount of agar was but 4 or 5 mg. On reference to No. 8 of Table 62 it will be seen that even 2.5 mg. of agar may cause a fatal shock. Undoubtedly, the results are influenced not only by the amount and state of the agar, and the rate of injection, but also by the varying resistance of the guinea-pigs.

A striking feature in connection with the tests detailed in Table 60, and elsewhere, was a period of incubation or quiet following the injection. Sometimes no effect was noticeable for fully 2 minutes, and then the characteristic symptoms of anaphylactic shock developed. Also, it is to be noted that the speed of injection is an important factor in this work just as it is in all other attempts at producing shock, regardless of the agent employed. The rapid injection of the high concentration is responsible for the sudden and atypical symptoms and findings in No. 4, Table 60. With this one exception the symptoms and findings in the other test animals were those of typical anaphylactic shock.

TABLE 61
INJECTIONS OF AGAR SOL-GEL (0 C.) INTO GUINEA-PIGS

Exper.	Guinea-Pig		Agar Sol-Gel		Result
	No.	Weight	c.e.* (intravenously)	Injection Time (sec.)	
A	1	209	4	30?	Severe. Nil for 3 minutes
	2	220	5	"	Near-kill. Nil for 2 minutes 30 seconds
	3	206	"	15?	7'35". Atypical shock, blood fluid, no clot
	4	235	6	?	Very slight. Nil for 3 minutes
	5	210	5	60	Very slight
	6	180	"	30	Very severe. Nil for 2 minutes
	7	182	"	15	2'. Atypical shock, clot doubtful
B	8	212	5	30	Moderate. Nil for 2 minutes
	9	203	"	"	5'20". Nil for 2 minutes; typical shock and autopsy
C	10	207	"	"	Very slight. Nil for 3 minutes
	11	297	"	"	1'40". Atypical shock
	12	320	"	"	Very slight. Nil for 4 minutes
	13	320	"	"	Nil
D	14	302	"	60	Slight. Nil for 3 minutes
	15	277	7.5	75	Slight. Nil for 2 minutes
	16	265	5	60	Nil
	17	250	"	"	2'15". Atypical shock
	18	252	"	"	Moderate. Nil for 2 minutes

* The number of milligrams of dry agar injected corresponds to the number of cubic centimeters in the fatal cases, Nos. 3, 7, 9, 11, and 17; per kilo of body-weight, this corresponds to 24, 27, 24, 17, and 20 mg.

In Table 61 is recorded a series of results with sol-gel mixtures, the diluent being distilled water.

The method of preparation was exactly the same as before. The mixture consisted of 1 part of sol and 4 parts of distilled water; it was shaken for 1 minute in Expers. A and B, and for 5 minutes in Expers. C and D. After icing for 1 hour, the perfectly clear liquid was injected directly, without any shaking, in Expers. A and D; and with vigorous shaking for 1 minute in Exper. C.

Exper. A was intended to show the effect of the rate of the injection on the development of the shock. Two different mixtures, prepared in exactly the same way, were employed; one was used for Tests 1 to 3, and the other for Tests 4 to 7. It will be seen that very slow injections, requiring 1 minute or more, have little or no effect.

The controls given in Part IX may serve for a like purpose at this place; they show that the injection of 5 c.c. of distilled water per 100 gm. of body-weight is without effect; the injection of 7.5 c.c. per 100 gm., when given very slowly, is likewise without effect, but if given rapidly, it may cause an atypical shock similar to those of Nos. 3, 7, and 11, Table 61.

In Exper. B, the first test (No. 8) was made without shaking the mixture, whereas for Test 9 the same mixture, after standing in the room for 1 hour, was thoroughly shaken before injection. The fatal shock in this case is probably due to individual variation in guinea-pigs rather than to the treatment of the mixture. A perfectly typical anaphylactic shock was obtained in this test.

In Exper. C, Test 10 was made at once after the usual icing for 1 hour, the mixture being thoroughly shaken. It was then returned to 0 C. for 3 hours and when retested on No. 11 it rapidly killed. On being tested again, 17 minutes later, it had little or no effect. These results again point to the individual resistance of the guinea-pigs.

In Exper. D, the first two tests were made after icing for 1 hour; the third (No. 16) was tried after icing for 2 hours more. The mixture was then placed at 37 C. for 24 minutes and when tested on No. 17 it gave a fatal result. Retested 6 minutes later (No. 18) it again failed to give more than a moderate effect. These variations, it will be seen, cannot be accounted for except on the basis of individual susceptibility on the part of the test animals.

The atypical shock and findings observed in 3 of these animals must not be interpreted as due to an entirely different mechanism (that is, agar embolism) from that of the characteristic anaphylactic shock. Rather, it must be looked upon as a reaction of the same type but moving at an increased speed and with greater violence. Anaphylatoxin-production and fibrin-coagulation are essentially twin reactions, in which presumably a certain labile protein constituent of the plasma (or serum) of the type of fibrinogen is involved. Instant paralysis of respiration means an absence of symptoms such as peripheral irritation, spasms, and convulsions; it likewise means incomplete distention of the lungs. In extremely rapid death, a clot may be found in the heart, while in slow death it is doubtful or absent. It is noteworthy that the blood on removal to a test tube may remain perfectly noncoagulable, as was the case in No. 3, in which it remained fluid for 24 hours; in others, the blood may show a slight viscosity,

which soon passes away and leaves it perfectly fluid, or else goes on to form a minute clot, the remainder of the blood being unchanged.

The typical shock, it is seen, is ushered in with a latent period of from 2 to 3 minutes; then follows the usual train of anaphylactic symptoms—peripheral irritation, dyspnea, spasms, convulsions. These were particularly noted in Nos. 1, 2, 6, and 9, Table 61. Death in such cases always occurs in from 5 to 7 minutes, this being the sum of the latent and symptomatic periods.

The latent period has an important bearing on the mechanism of the intoxication. When an anaphylatoxic serum is injected, the latent period is short, rarely exceeding 30 seconds, which means that the ready-made poison acts immediately. When, however, the sol-gel mixture is injected, the poison must be made within the animal, and this takes an appreciable length of time. It can be shown by in-vitro experiment that agar can and does produce, at 37 C., a fatal dose of poison in less than 2 minutes. With the large amount of blood in the animal, it is obvious that several lethal doses may be produced in the same time and even in less. As bearing on this point reference is made to Table 65. It may be added that a latent period is seen in all similar intoxications, even in poisoning with minimal doses of potassium cyanid.

In the typical agar intoxications, therefore, the effects cannot be ascribed to the agar itself, but to the anaphylatoxin which is made within the blood of the animal as a result of the disturbance set up by the presence of an alien substance. In other words, the agar initiates or intensifies the reaction proper which leads to poison-production. It exerts a force comparable to the pull of a trigger; the blood vessel is the tube carrying the explosive charge. The reaction which takes place will be shown to be essentially identical with that which occurs in a sensitized animal when shocked (true anaphylaxis); and further, it will be found to be identical with that which occurs in vitro when anaphylatoxin is made in normal serum.

The relatively meager results obtained with distilled water as a diluent (Table 61) led to further work with salt solution in the hope of securing more positive effects and a better understanding of the reaction involved. It will be seen from Tables 62 and 63 that these attempts proved most satisfactory.

The experiments recorded in Table 62 were made with a sol-gel mixture in which salt solution was used in place of distilled water.

They are designated as B, C, and D and were made on the same day as the experiments with corresponding designation in Table 61. The same sol-gel was used and the conditions were the same, the experiments being made in parallel. The two tables are therefore directly comparable except in Exper. D, where the mixture used for No. 7 was iced for 2 hours longer than in the corresponding test (No. 16); Nos. 8 to 10 are not duplicated in the preceding table.

TABLE 62
INJECTION OF AGAR SOL-GEL (0 C.) INTO GUINEA-PIGS.
(DILUTIONS MADE WITH SALT SOLUTION 1:4)

Exper.	Guinea-Pig		Agar Sol-Gel		Result
	No.	Weight	c.c. (intravenously)	Injection Time (sec.)	
B	1	200	5	30	Severe. Nil for 3 minutes
	2	206	"	"	5'. Nil for 2 minutes. Typical shock and autopsy
C	3	215	"	"	Moderate. Nil for 1½ minutes
	4	290	"	35	Very slight. Nil for 2 minutes 45 seconds
	5	275	"	30	6'10". Nil for 2½ minutes. Typical shock and autopsy
	5a	310	"	"	Nil
D	6	250	"	60	Death after 2 hours. Nil for 1 minute
	7	260	"	"	Slight. Nil for 2 minutes
	8	265	5*	"	12". Nil for 2 minutes, then typical shock and autopsy
	9	265	5*	"	Very severe. Nil for 2 minutes 30 seconds
	10	260	2.5	30	Nil

* For Tests 8 and 9 the sol-gel mixture was diluted with an equal volume of distilled water. In the fatal cases, Nos. 2, 5, 6, and 8, the amount of dry agar, per kilo of body weight, was 24, 18, 18, and 9.4 mg., respectively.

A comparison of Tables 61 and 62 shows that the mixtures with salt solution apparently do not give an atypical shock such as was obtained with those in which distilled water was used. The symptoms and findings are those of typical anaphylactic shock. Particularly noteworthy are the last three tests. For Tests 8 and 9 the sol-gel salt mixture was diluted with an equal volume of distilled water; 5 c.c. of this, tested at once, proved fatal in No. 8, and the test repeated 45 minutes later gave a very severe shock (No. 9), while the undiluted mixture was without effect in No. 10. The dilution of the mixture seemed to have a beneficial effect, and it may be added that similar favoring action has been observed with other agents.

With reference to these tests, it is important to note that even as small an amount as 2.5 mg. of agar is capable of producing a fatal anaphylactic shock (No. 8). Doerr and Russ¹ found colloidal silicic acid to kill in dose of from 5.6 to 7 mg. While Friedberger and Tsuneoka² were unable to produce death with kaolin in dose less than 15 mg. Consequently, it is seen that apparently so harmless a substance as agar, in the proper state, can be more toxic than either of these colloids.

The toxicity of kaolin-treated serum was at one time ascribed by Friedberger,³ not to the formation of anaphylatoxin, but to the suspended kaolin, and he explained the effect on the temperature after injection as due to injury of the endothelial lining of the blood vessels by the sharp corners of the kaolin, and death he considered as due to embolism. The fact that inactivated treated serum with kaolin had no effect was explained with equal facility by assuming that the sharp corners had become coated with coagulated albumin. Eventually, with Tsuneoka he realized that the kaolin toxicity was not mechanical, and to account for the effects he felt obliged to imagine that they depended on the absorption of certain constituents of vital cells!

The fact that the 10% sol-gel mixture (D. No. 8) killed in a dose containing but 2.5 mg. of agar, suggested a comparative trial of 10, 15, and 20% mixtures. Two such series of trials were made and the results are given in Table 63. The mixtures were prepared by adding 6 c.c. of the 37 C. sol to 54, 33.6, and 24 c.c. of salt solution, respectively, the diluent having been previously warmed to 37 C. After thorough shaking for 5 minutes, the mixtures were placed in cracked ice for 1 hour; the tests A and B were then made.

The smallest amount of agar which proved fatal in these experiments was 3.75 mg. (Nos. 2, 3, 9, and 14). Possibly better results are obtainable with fresh mixtures iced for only half an hour. On the whole, the results were much better than those in previous trials; of the deaths, only one was atypical.

It would be difficult to say which dilution gave the best results. It was believed at the time that the variations seen, for example, in Exper. B, Nos. 9, 10, and 11, were due to a loss in toxifying power

¹ Wien. med. Wchnschr., 1912, 25, p. 338.

² Ztschr. f. Immunitätsf., 1913, 20, p. 405.

³ Centralbl. f. Bakteriöl., R., 1913, 57 (Beiheft), p. 242; that is, Bericht nb. d. 71. Tagung d. Fr. Ver. f. Mikrobiöl. in Berlin, 1913. Ibid., 1912, 54 (Beiheft. ot. Tagung), p. 251.

because of the time which had elapsed between the tests; these tests were made 18 and 11 minutes apart. A similar condition was seen in Exper. C, in which the same sol-gel mixture was tested after icing for 36 and 76 minutes. It is more likely in view of results such as were presented in Table 45, that these variations were due to the inconstant behavior of the animals rather than to the slight changes mentioned.

The rate of injection, however, seems to be a factor of importance. Thus, in Test 4, the guinea-pig was given 2 injections of 5 c.c. each, 2 minutes apart. The very slight effect produced in this case is to be compared with the results of the tests immediately preceding and following (Nos. 3 and 5); the former, which was made 28 minutes before, killed, and the latter, made 11 minutes after No. 4, gave a severe shock. It would seem from this as if the divided dose exerted a distinctly minimal action. The result is in line with previous observations that large amounts slowly injected may be less dangerous than smaller quantities rapidly injected. Similar results are known to occur when peptone is injected in divided doses.

TABLE 63
INJECTION OF AGAR SOL-GEL (0 C.) INTO GUINEA-PIGS
(DILUTIONS OF 10, 15, AND 20% IN SALT SOLUTION)

Exper.	Mix- ture	Guinea-Pig		c.c. (intraven- ously)*	Result
		No	Weight		
A	1:4	1	250	5	8'50". Nil for 2 minutes, typical shock and autopsy
	1:5.6	2	265	"	9'30". Nil for 2 minutes, typical shock and autopsy
		3	255	"	8'45". Nil for 4 minutes, typical shock and autopsy
		4	265	10	Very slight
		5	250	5	Severe. Nil for 3 minutes
	1:9	6	248	"	Slight. Nil for 4 minutes
		7	256	10	4'40". Nil for 2 minutes
B	1:4	8	285	5	6'25". Nil for 1½ minutes, typical shock and autopsy
	1:5.6	9	297	"	13'. Atypical shock, blood fluid, no clot
		10	315	"	Severe. Nil for 1½ minutes
		11	310	"	Slight. Nil for 2½ minutes
	1:9	12	286	10	5'10". Nil for 1½ minutes, typical shock and autopsy
		13	245	5	Slight. Nil for 3 minutes
C	1:5.6	14	320	"	5'30". Nil for 3 minutes, typical shock
		15	320	"	Moderate

* The injection time for the tests was 1 minute for 5 c.c. and 2 minutes for 10 c.c.
The amount of dry agar, per kilo of body weight, in the fatal cases ranged from 11 to 20 mg.

INJECTION OF RABBITS

Only 2 tests were made to ascertain the effect produced by agar when injected intravenously into rabbits. A more extended effort would have given in all probability as positive results as in the case of the guinea-pigs. However, the findings were sufficient to indicate that agar does exert a similar action.

The sol-gel mixtures for these experiments were made by adding 1 part of sol to 9 parts of salt solution, both previously having been kept at 37 C. After vigorous mixing for 5 minutes, the sol-gel was placed in cracked ice for half an hour. This dilution, as previous experiments had shown, was fatal to guinea-pigs in dose of about 40 c.c. per kilo. The same mixture as used for Exper. 2 was injected in dose of 10 c.c. into Rat 16 (Table 64). This dose, which represents 60 c.c., or 30 mg. of agar per kilo, produced but a moderate effect in the rat, whereas Rabbit 2 died after receiving approximately half this amount. It would seem therefore that the rabbit holds an intermediate place between the guinea-pig and the rat.

Exper. 1.—A rabbit weighing 1450 gm. was given 30 c.c. of the mixture (=20.5 c.c. per kilo); in 2 minutes it showed marked peripheral irritation, but otherwise nothing.

Exper. 2.—The rabbit in this test weighed but 350 gm. It received 11.6 c.c. of the mixture, which represent 33.1 c.c. or 16.6 mg. of agar per kilo. An atypical shock resulted; the animal was at once on its side, with head drawn back, but not rigid; the respiration became deep and in 3 minutes dyspnea appeared; death occurred in 9 minutes, 30 seconds. The autopsy, made 4 minutes after death, showed partial distention of lungs, vigorous heart beat, and the blood perfectly fluid. Blood drawn from the heart and placed in a test tube clotted in 11 minutes. At 31 minutes after death the heart was still beating and no clot was present. Consequently, in this, as in many similar tests, the blood transferred from the heart to a test tube, by means of a wet syringe, clotted before that which was left in the heart. This method is therefore of little value in ascertaining whether or not the blood of a shocked animal is incoagulable. A better procedure is that which will be discussed in Part VII.

INJECTION OF RATS

The behavior of rats in response to injections of agar presents an interesting paradox corresponding with that observed in sensitized rats injected with dilute antigen or with distilled water (Part IX). It will be recalled that normal rat serum on treatment with agar yields the most powerful anaphylatoxin known, its lethal dose being from 0.2 to 0.25 c.c. per 200 gm. of guinea-pig; furthermore, it has been shown in Part V that such highly toxic sera may be injected, intravenously, in

relatively large doses into rats without producing fatal results. Weight for weight, the rat seems to tolerate more than 100 times as much of the poison as does the guinea-pig; that is, a 150-gm. rat survived 75 guinea-pig lethal doses.

On the basis that 10% of the body weight is blood, a rat weighing 150 gm. would carry 15 c.c. of blood, or 9 c.c. of plasma; were this toxified to the same degree as occurs *in vitro* with serum, it would yield from 36 to 45 guinea-pig lethal doses, an amount which represents from one-half to two-thirds of the dose named as nonfatal for the rat. Consequently, an additional amount of poison must be produced within the body in order to cause death, and this means, if the plasma is considered as the sole source of the poison, that the real matrix is present in greater amount in the plasma than in the serum. That such actually is the case is indicated by the high, pre-coagulation toxicity of blood as compared with that of serum made from the same blood. For example, 2 c.c. of rabbit blood (= 1.2 c.c. plasma) just before coagulation, may be fatally toxic to a guinea-pig, whereas the serum may not be poisonous in dose of 6 c.c., 5 times the amount of the plasma which did kill. Whether the very labile fibrinogen or some constituent of the blood cells is responsible for this increased toxicity of whole blood remains to be demonstrated.

Clearly, in view of the large amount of poison necessary to kill, it should be very difficult to produce anaphylactic death, either by the injection of agar into normal rats, or by the specific anaphylactic shock induced by injection of an antigen into a previously sensitized rat. This was actually found to be the case for both of these procedures. The rat is remarkably tolerant for anaphylatoxin, whether this be injected preformed, or whether it be made *in vivo* as a result of treatment with agar or antigen.

The results of injections of agar into rats are given in Table 64. For these experiments, the sol-gel mixtures, prepared with salt solution in the usual way, were iced for half an hour before use. The injections were made into the femoral vein. It will be noted that a number of the tests apparently had no effect, the respiratory disturbance being very slight or absent; others resulted in distinct manifestations of shock. Of the 3 deaths, 1 (No. 13) was perfectly typical both as regards symptoms and autopsy findings. The amount of agar given in the fatal injections (Nos. 5, 7, and 13) was, respectively, 10, 3.8, and 5 mg.; or better expressed, it was 80, 27, and 32 mg. per kilo of body

weight. It is evident from these tests that rats react to agar much less than do guinea-pigs, even tho the dose is relatively larger.

Inasmuch as the sol-gel mixture used for Tests 1 to 3 had very little effect, it was tried out on a 258-gm. guinea-pig in dose of 5 c.c. and was found to yield but a mild shock. Accordingly, a new solution was prepared and this, when tested at once on a 310-gm. guinea-pig in like dose of 5 c.c., produced a typical anaphylactic shock and death in 5 minutes. This active mixture was then used for Tests 4 and 5. Similar controls made after Tests 9 and 10 gave only mild effects in guinea-pigs.

TABLE 64
INJECTION OF AGAR SOL-GEL GEL (0 C.) INTO WHITE RATS
(DILUTIONS OF 10, 15, AND 20% IN SALT SOLUTION)

Mixture	Rat		Agar Sol-Gel Gel		Result
	No.	Weight	c.c. (intravenously)*	Injection Time (min.)	
1:4	1	130	2	$\frac{1}{2}$	Very slight. Polypnea Peripheral irritation; irregular respiration on side Irregular respiration Irregular respiration 2%. Atypical shock
	2	135	3	1	
	3	110	4	$1\frac{1}{2}$	
	4	135	5	1	
	5	125	10	$1\frac{1}{2}$	
1:5.6	6	135	3	1	Nil 6/10%. Atypical shock Nil Increased respiration Increased respiration
	7	140	5	1	
	8	160	"	$\frac{1}{2}$	
	9	125	"	$\frac{3}{4}$	
	10	110	"	1	
1:9	11	132	"	1	Nil Nil 5/30%. Nil for 2 minutes 30 seconds, then typical shock and autopsy Increased respiration Increased respiration Increased respiration with some dyspnea Increased respiration Practically nil
	12	127	7.5	1	
	13	155	10	$\frac{1}{2}$	
	14	124	"	$1\frac{1}{2}$	
	15	130	"	$1\frac{1}{2}$	
	16	165	"	$1\frac{1}{2}$	
	17	150	13	$1\frac{3}{4}$	
	18	145	15	2	

* In the fatal cases, Nos. 5, 7, and 13, the amount of dry agar per kilo of body weight was 80, 27, and 32 mg., respectively.

The mixture employed for Tests 11 to 13, was first tested on a 320-gm. guinea-pig, which it killed in dose of 10 c.c. in 10 minutes, 30 seconds; a similar control preceded Tests 14 and 15 and here, also, the mixture was shown to be active — a 320-gm. guinea-pig responding to 10 c.c. with typical shock and death in 5 minutes, 30 seconds. Lastly, it may be stated that the mixture used for No. 16 was injected 40 minutes before into Rabbit 2; the dose of the mixture, per kilo, in the case

of the rabbit was 33 c.c. (= 16.5 mg. of agar), while that for the rat was 60.6 c.c. (= 30.3 mg. of agar). It will be seen that the rat, which received nearly twice as much of the agar as the rabbit, showed but slight effects, while the latter died. It may be further pointed out that in Test 18, the rat received 15 c.c. of a similar mixture, the injection being given in 2 minutes with practically no effect; the amount of agar injected in this case corresponded to 51.7 mg. per kilo.

There can be very little doubt but that the susceptibility of the three species tested varies as regards the injection of agar. Selecting the lowest fatal doses found, one may compare them, per kilo of animal, as follows:

Guinea-pig	9.4 mg. of agar
Rabbit	16.5 mg. of agar
Rat	27.0 mg. of agar

It is seen that the toxicity for the three animals is relatively 1:2:3. A similar relative resistance will be shown to exist in the case of poisoning with Witte's peptone (Part VII), and this fact supports the view that the intoxications by agar and by peptone are of exactly the same nature. The transfusion experiments will furnish direct evidence of this identity.

TRANSFUSION OF BLOOD OF RATS SHOCKED WITH AGAR

The previous set of experiments, taken by itself, might not be considered to bear more than a resemblance to anaphylatoxic poisoning. At first sight, the symptoms (except in No. 13) suggested little or no relation to those of shock in the guinea-pig. But it must be remembered that the rat does not behave like the guinea-pig when injected with anaphylatoxin; and, it will be shown later that this difference is equally marked when specific anaphylactic shock is attempted.

It had previously been shown that the rat could tolerate an enormous dose of anaphylatoxin and, in view of the known ease with which rat serum was toxified with agar, it was reasonable to believe that the same poison was produced *in vivo*. When everything is considered, the previous experiments strengthen this view. However, actual proof of the formation and presence of anaphylatoxin in the blood of the treated rats was required, and this could be supplied in but one way: by transfusing, at maximal speed, some of the rat blood to a normal guinea-pig.

It was pointed out in connection with Table 64 that, it being assumed the plasma responds to agar in the same way and to the same extent as serum, it is possible for a rat of 150 gm. to develop from 36 to 45 guinea-pig lethal doses. After an injection of 5 or 10 c.c. of the agar mixture the total volume of the blood would be 20 and 25 c.c., respectively. The transfusion of 2 c.c. of such diluted blood implies a possible transfer of from 3.6 to 4.5 guinea-pig lethal doses in the case of the 20-c.c. bulk, and of from 2.9 to 3.6 guinea-pig lethal doses in the case of the 25-c.c. bulk. Or, in round numbers, the transfusion of 2 c.c. of the blood would mean a possible transfer of 3 guinea-pig lethal doses, and, allowance being made for some in-vivo destruction of the poison, there should still be left enough to give a fair indication of its presence. This view was amply confirmed by the experiments recorded in Table 65. It may be added at this point that the same method applied to the peptone shock in rats showed that actually somewhat more than 7.5 guinea-pig lethal doses were present (Part VII).

Preliminary experiments on the transfusion of the blood of normal rats (Table 66) showed that, with fair speed, 2 c.c. of the rat blood could be transfused without producing much, if any, effect in the recipient. Hence, essentially the same negative result should follow the injection of the blood of rats injected with agar, provided the transfusion was made at the earliest possible moment. After the agar had had a chance to act in vivo for 2 or 3 minutes, which represents the latent period to which attention has been called, the blood should show the presence of the poison. And, further, in view of the fact that recovery from the shock was usually rapid, a fact implying the disappearance of the poison, it was expected that transfusion after 5 or 6 minutes would show a decrease or absence of the poison.

In Table 65 the tests are arranged according to the 'total time' required for the injection of the agar and for the transfusion of the blood; that is, the actual time from the start of the injection of the rat until the end of the injection of the guinea-pig. By 'transfer time' is meant the interval from the entrance of the syringe into the heart of the rat until its withdrawal from the vein of the guinea-pig. It represents, therefore, the maximal time any portion of the blood was in the syringe. This transfer time must be kept as short as possible, otherwise in-vitro toxification may occur (see Table 66). The difference between the 'total' and the 'transfer' time represents approximately the reaction time within the rat.

It will be seen that with a reaction time of about 1 minute (Nos. 8 and 9) the dose of blood employed contained very little poison. It is possible that a dose of 4 c.c. at this stage would have proved fatal.

With the reaction time increased to 2 minutes and especially to 3 to 7 minutes, the presence of anaphylatoxin is demonstrable either by severe or by fatal shock. With this time increased to 11 minutes, the toxicity of the transfused blood is apparently reduced to that of normal blood (Nos. 14 and 15).

TABLE 65
THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN RATS INJECTED WITH AGAR (1:9)

Exper.	Rat			Weight Guinea-Pig*	Total Time	Trans-fer Time	Result in Guinea-Pig
	No.	Weight	Agar (c.c.)				
A	1	170	10	200	2'35"	25"	19'. Typical shock and autopsy
	2	164	"	205	3'	25"	Good shock
	3	140	"	171	5'25"	45"	Practically nil
	4	160	"	175	6' 5"	45"	3' 5". Quiet shock
	5	148	"	191	6'25"	25"	4'45". Typical shock and autopsy
	6	155	"	184	6'30"	30"	3'30". Typical shock and autopsy
	7	160	"	177	6'50"	30"	5'50". Typical shock and autopsy
B	8	125	5	197	1'10"	15"	Slight
	9	148	"	148	1'30"	25"	Very slight
	10	145	"	202	3'50"	23"	4'25". Typical shock and autopsy
	11	130	"	190	4'20"	25"	3'55". Typical shock and autopsy
	12	115	"	181	4'55"	30"	Moderate
	13	155	"	174	7'20"	30"	4'25". Typical shock and autopsy
	14	168	"	198	11'35"	65"	Slight
	15	170	"	185	11'40"	30"	Practically nil

* In every test 2 c.c. of heart blood were transferred to the guinea-pig.

It is significant that in both series of tests, with a reaction period of 4 minutes 40 seconds, and 4 minutes 25 seconds (Nos. 3 and 12), the transfused blood should be considerably less toxic than in the periods immediately preceding and following. It would seem as if the first production of poison was followed by a distinct drop or reversion, which in turn was followed by a second rise in toxicity, which gave way in turn to an atoxic stage. Whether the first drop in toxicity was actual, or was merely an accident due to the chance resistance of the guinea-pig used, can only be determined by repeated trials. The fact that a similar drop was observed in corresponding transfusions from rats injected with peptone (Chart 11) would seem to indicate that it expresses something more than a chance resistance.

The results given in Table 65 are expressed graphically in Chart 9, in which the abscissæ represent the reaction time.

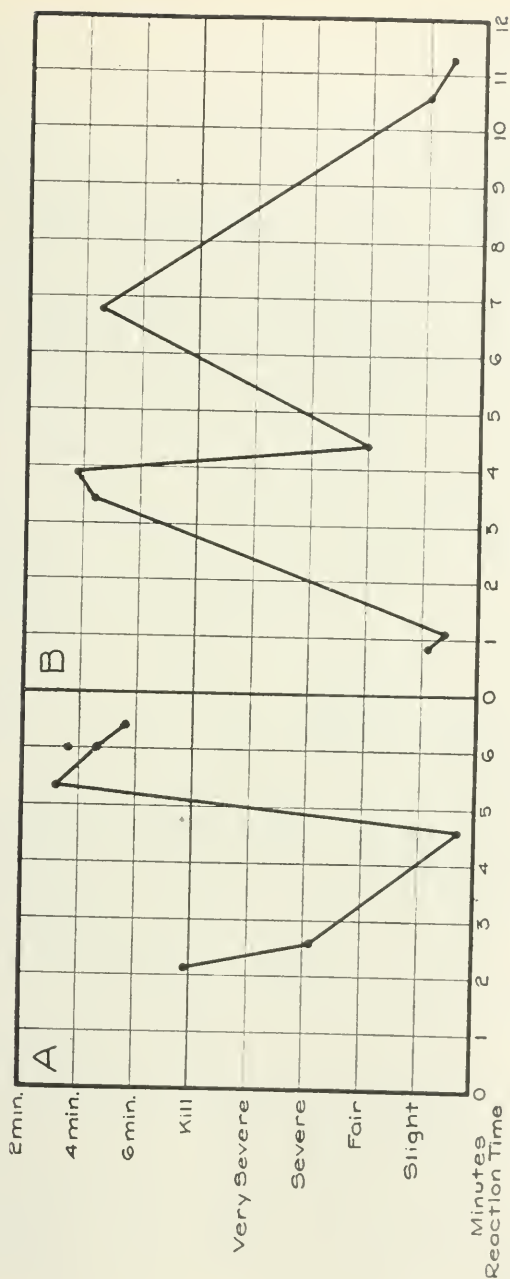


Chart 9. The in-vivo production of anaphylatoxin in rats injected with agar (Table 65).

These experiments establish beyond doubt that a poison, identical in action with the in-vitro anaphylatoxin, is produced in rats injected with agar. And, further, such poison is formed tho the rats themselves show but little effect. The autopsy findings in the guinea-pigs were those of typical anaphylactic shock with the single exception of No. 4. The fact that 2 c.c. of the blood is fatal may be taken to indicate that the injected rat contains at least 10 guinea-pig lethal doses.

It is deserving of mention that the hearts of the rats used in these tests when examined about 10 minutes after death were still beating, and the blood was perfectly fluid, with no evidence of clot. Such blood transferred to a test tube clotted in about 3 or 4 minutes.

It should be added that the sol-gel mixtures used for these tests were prepared in the usual way. One part of the sol, at 37 C., was mixed with 9 parts of the salt solution and thoroughly shaken for 5 minutes; the mixture was then placed in cracked ice for half an hour. Three such mixtures were used in Exper. A and three in Exper. B. The injections were made into the femoral vein in the rats and into the jugular vein in the guinea-pigs. The animals were immobilized, side by side, and the veins exposed before beginning the test proper. The injection time for 10 c.c. of the agar mixture varied from 1 to 2 minutes; for 5 c.c. it ranged from 15 to 40 seconds.

Transfusion of Blood of Normal Rats to Guinea-Pigs.—In Table 66 will be found the results of the transfusion of the blood of normal rats. These tests serve as controls for the experiments recorded in Table 65 and for similar transfusions. To be strictly correct, these controls should have been made with rats which had received injections of 5 or 10 c.c. of salt solution, and with reaction times corresponding to those of the experiment proper. Two such tests with egg white diluted with distilled water are recorded in Part IX. A rat was used for each experiment; after exposure of the heart, the blood was drawn up into a syringe and at once injected into the jugular of a guinea-pig.

The most important factor in these transfusions is the transfer time. It will be noted that even 4 c.c. of blood can be transfused with little or no effect provided the transfer time does not exceed 50 seconds. With a transfer time of 1 minute (No. 6), this dose of blood caused a typical anaphylactic shock. This precoagulation anaphylatoxin develops more rapidly in rat blood than it does in rabbit or guinea-pig blood (see Part VIII). This is directly due to the more rapid clotting of the rat blood.

Transfusion of Blood of Guinea-Pigs Shocked with Agar.—The successful demonstration of the in-vivo formation of anaphylatoxin in rats which had received an agar injection led to a series of similar tests with guinea-pigs. It could hardly be expected that the results would be as good in view of the fact that guinea-pig serum toxifies but feebly; and, therefore, if this was equally true for the in-vivo reaction, the guinea-pig would contain but few lethal doses. For that reason it was deemed best to transfuse as large doses of blood as possible within a transfer time not exceeding 1½ minutes. Previous tests had shown that 5 c.c. of normal guinea-pig blood, drawn from the heart and kept in the syringe for 2 minutes, produced little or no effect on subsequent injection (Part VIII). To be correct, the controls should be made with guinea-pigs injected with a like amount of salt solution.

TABLE 66
CONTROL TRANSFUSIONS OF BLOOD OF NORMAL RATS TO GUINEA-PIGS

Guinea-Pig		Rat Blood		Result
No.	Weight	c.c.	Transfer Time (min.)	
1	170	2	50	Practically nil
2	176	"	40	Very slight
3	172	"	45	Practically nil
4	205	4	45	Slight
5	210	"	48	Practically nil
6	187	"	60	Death in 3/5". Typical shock and autopsy

For the experiment given in Table 67, the agar mixtures were prepared in exactly the same way as those used for the rat experiments. The sol-gel dilution was injected in dose of 10 c.c., and immediately thereafter the heart was exposed and 6 c.c. of blood withdrawn and injected into a new guinea-pig. Dyspnea was present in each of the 4 donors at, or before, the time of section and, according to the results in Table 63, death was expected in 5 or 6 minutes. Hence, at least 1 lethal dose of poison was in evidence, and this poison was distributed in an amount of blood corresponding to about 10% of the body-weight, plus the volume of fluid injected, that is, in about 35 to 38 c.c.

It will be seen from Table 67 that but 1 of 4 trials was successful, which may be taken to indicate that only a small amount of poison was present in the blood. The fact that 6 c.c. did kill in No. 3, means that this animal had at the time about 5½ guinea-pig lethal doses in its blood. This, it will be seen, is considerably less than the amount pres-

ent in specific anaphylactic shock induced in guinea-pigs sensitized with egg white (Part IX), in which from 14 to 24 guinea-pig lethal doses were demonstrated.

In the tests given in Table 67, the reaction time was approximately the same in all, ranging from 4 minutes to 4 minutes 25 seconds. Because of the fall in blood pressure it was undesirable to lengthen this period, unless larger animals were used as donors, and this at the time was impracticable. It is possible that, at the 4-minute period, a decrease in the amount of poison occurs, such as has been noted in rats injected with agar or with peptone (Charts 9 and 11). To decide this point it will be necessary to make additional tests with a reaction time of from 2 to 3 and 5 to 8 minutes.

TABLE 67

THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIGS INJECTED WITH AGAR (1:9)

Donor Guinea-Pig		Agar (c.c.)	Weight of Recipient*	Total Time	Transfer Time	Result in Guinea-Pig
No.	Weight					
1	270	10	199	6'	1'35"	Very slight
2	275	"	200	5'	55"	Slight
3	255	"	195	5'15"	1'15"	8'45". Typical shock
4	280	"	175	5'35"	1'35"	Slight

* In every case 6 c.c. of heart blood were transferred to the recipient.

TRANSFUSION OF BLOOD OF RABBITS SHOCKED WITH AGAR

It has been pointed out in Part V that the rabbit, like the rat, tolerates large doses of agar anaphylatoxin, a fact which enables it to carry enormous amounts of this poison in vivo when the poison is developed as the result of injection of sheep corpuscles. It is indeed possible for a treated rabbit to have in its blood as much as 2000 guinea-pig lethal doses. Since in the rat, injections of agar result in the production of anaphylatoxin, it seemed as if the same reaction, tho perhaps less intensively, should occur in the rabbit, and if so it should be possible to demonstrate the formation of the poison by transfusion tests. It has been shown heretofore that the injection of agar in rabbits may cause typical anaphylactic shock and death, which if due to anaphylatoxin would imply the formation of considerably more than 1 guinea-pig lethal dose. The transfusion experiments seemed to be a simple matter since previous tests, some of which are given in Part VIII, showed that with attention to speed, as much as 10 c.c. of rabbit blood could be transferred without serious results.

Accordingly, transfusion experiments were made on 6 rabbits after they had received, intravenously, varying amounts of sol-gel salt mixtures.

The latter were prepared as heretofore; that is, the agar sol at 38 C. was added to 4 or 9 parts of 0.85% salt solution, and the mixture after thorough shaking for 5 minutes was placed in cracked ice for an hour or more. For each test, 2 c.c. of blood were drawn by heart-puncture, through the thoracic wall, and at once injected into the jugular vein of a guinea-pig; the transfer time—that is, from the start of the drawing of the blood until the end of the injection of the recipient—usually ranged from 12 to 20 seconds, tho occasionally it took 30 seconds. Before injecting the agar solution, as a control precaution, the toxicity of the heart blood was tested on guinea-pigs; 2 transfusions, each of 2 c.c. were made, and if the effects thus produced were but slight the injection of the agar was then carried out. The transfers then followed at intervals which are designated as 'total time'—that is, from the start of the agar injection until the end of the injection of the guinea-pig. The difference between the transfer and total times represents the reaction time during which the agar has acted on the rabbit blood. The agar injections in rabbits always caused more or less respiratory trouble but all 6 used for these tests recovered.

Exper. 1.—Two preliminary controls showed but slight effects. The rabbit (2250 gm.) was then injected with 45 c.c. of the iced sol-gel salt mixture (1:4), the injection time being 2 minutes 5 seconds. This dose represents 45 mg. of agar, or 20 mg. per kilo. The total-time transfers were made at 3'32", 5'17", 8'11", and 11'21", with but moderate results. Apparently, there was no marked increase in the toxicity of the blood.

Exper. 2.—The two preliminary tests showed moderate shock effects. The rabbit (2040 gm.) was then given 40 c.c. of a sol-gel salt mixture (1:9), the injection time being 1 minute 35 seconds. The dose given represents 9.8 mg. of agar per kilo. The total-time transfers were made at 3' 9", 6' 5", 8' 7", 11' 13", and 16' 58". The second of these resulted in a typical shock and death in 4 minutes, 10 seconds; the other tests were apparently of increased severity compared with the preliminary tests.

Exper. 3.—A single preliminary test gave but slight effects. The rabbit (900 gm.) then received 9 c.c. of a sol-gel salt mixture (1:4), the injection time being 1 minute. This dose corresponds to 10 mg. of agar per kilo. The total-time transfers were made at 2' 30", 5' 30", 9' 45", 13', 16', and 22'. Of these, the third resulted in typical shock and death in 2 minutes, 27 seconds, and the fourth caused a very severe shock; the others had but slight effects.

Exper. 4.—No preliminary tests were made. The rabbit (2380 gm.) was given 40 c.c. of a sol-gel salt mixture (1:4) in 2 minutes, 33 seconds. The amount of agar was 17 mg. per kilo. The transfer made at 4 minutes, 9 seconds proved fatal in 6 minutes, 29 seconds; that at 6 minutes, 42 seconds gave a very severe shock, as did also that at 14 minutes, 50 seconds. Three other transfers at 10' 50", 19' 20", and 24' 30" had but slight effect.

Exper. 5.—No preliminary tests were made. The rabbit (2340 gm.) received at once 23 c.c. of a sol-gel salt mixture (1:4) in 1 minute, 50 seconds. The amount of agar given was 10 mg. per kilo. The transfer at 18 minutes caused typical shock and death in 3 minutes 50 seconds, while that at 22 minutes was severe. The 5 other tests made at 3, 6, 10, 14, and 26 minutes had slight or no effects.

Exper. 6.—See Table 68. After 2 preliminary tests (Nos. 1 and 2) the rabbit of 2300 gm. was given 23 c.c. of a sol-gel salt mixture (1:4), this amount representing 10 mg. of agar per kilo. It will be seen from the results of this experiment as tabulated, that apparently the toxicity of the blood had been increased after the injection of the agar suspension since 4 of 9 tests proved acutely fatal. Typical acute shock was developed in these fatal cases.

On summing up the 6 experiments it will be found that 8 out of 37 tests, or 21.6%, were fatal, the amount of blood transferred in each case being but 2 c.c. The number of deaths could have been materially increased by the injection of a larger amount of blood, but this was inadvisable because of preliminary transfusion tests with rabbits given injections of plain salt solution. Such tests were made with the object of having rigid controls for the agar experiments proper which were to follow.

TABLE 68
IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN RABBIT INJECTED WITH AGAR (1:4) EXPER. 6

Guinea-Pig		Rabbit Heart Blood			Result in Guinea-Pig
No.	Weight	c.c.	Total Time	Transfer Time	
1	215	2		33"	Slight
2	213	"		15"	Slight
3	207	"	4'18"	13"	5'52"
4	195	"	7'22"	27"	Slight
5	191	"	12' 8"	28"	2'47"
6	197	"	16'40"	27"	Very slight
7	205	"	22'25"	20"	4'50"
8	195	"	28'23"	18"	Slight
9	187	"	33'20"	20"	3'55"
10	187	"	38'49"	10"	Very slight
11	186	"	47'10"	15"	Fair

In the first control of this type, a rabbit of 1076 gm. received intravenously in 2 minutes, an injection of 20 c.c. of iced salt solution. The total-time transfer at 3 minutes, 55 seconds of 5 c.c. of heart blood caused typical shock and death in 4 minutes, 35 seconds, while a like amount transferred at 5 minutes, 37 seconds had but slight effect. Transfers of 3 c.c. were then made at 14 minutes and at 16 minutes, 30 seconds, which had also slight or moderate effects. Inasmuch as this experiment seemed to show that the injection of salt solution resulted in the production of some poison, similar tests were made with 3 other rabbits, the amount of blood transferred being reduced, however, to 2 c.c.

In a second control a rabbit of 1068 gm. was given 10 c.c. of a salt solution in 55 seconds, and 2 c.c. of blood transfused at 2 minutes, 37 seconds and 6 minutes, 37 seconds had but very slight effect. Similarly, in the case of a third control rabbit of 2050 gm., which received 40 c.c. of the salt solution in 1 minute, 55 seconds, total-time transfers at 3' 45", 6' 45", 10' 30", and 13' 20" caused very slight or no disturbance. Likewise, transfusions from a fourth control rabbit of 2078 gm. injected with 40 c.c. of the salt solution, gave no effect at the 4' 10" and 9' 10" periods. It will be seen, therefore, that 8 transfusions, each of 2 c.c., from 3 rabbits, caused relatively little or no disturbance and this fact led to the adoption of this dose of blood in the experiments with agar.

The control transfusions after injection of salt solution would seem to justify the belief that the results obtained with like transfusions after the injection of sol-gel salt mixtures were due to anaphylatoxin produced in vivo. Strictly speaking, however, this conclusion is open to question in view of the wholly unexpected results which were obtained with 3 rabbits in tests made on the same days as the preceding. The direct transfusion of 2 c.c. of heart blood from these caused typical shock and death (Table 69). These results were the more surprising since at various times during the past year or two, transfusions of from 5 to 10 c.c. of normal rabbit blood were found to be without effect. They show clearly that the so-called normal animal may possess blood of varying toxicity. These animals seemed to be perfectly healthy, and had never been used for experimental work. The rabbits which received injections either of agar or of salt solution, were undoubtedly in a similar, tho not as marked, state of autotoxicity, and for that reason the results obtained must remain in suspense.

The cause of this unusual toxicity cannot be definitely established. It certainly cannot be ascribed to malnutrition; the possibility of an intercurrent unrecognized epidemic may be conceded, tho highly improbable because of the perfect condition of the stock animals. The fact that these experiments were made in mid-winter (January) might suggest a seasonal state of the blood, but this was offset by the records of the previous January, when the harmlessness of rabbit blood had been first established. The only essential difference would seem to be in the age and size of the animals. For the foregoing tests, young rabbits of from 1 to 2 kilos were used, whereas formerly considerably larger animals were employed.

TABLE 69
CONTROL TRANSFUSIONS OF BLOOD OF NORMAL RABBITS TO GUINEA-PIGS; SERUM TOXICITY

Rabbit		Guinea-Pig		Rabbit Heart Blood		Result in Guinea-Pig
No.	Weight	No.	Weight	c.c.	Transfer Time (min.)	
1	1335	1	206	2	22	5'
2	2275	2	200	"	15	5'40"
		2a	205	"	15	5'
3	2060	3	195	"	12	3'
		3a	202	"	12	3'10"
"				Serum		
		4	195	1.0		2'20"
		5	193	1.0		5'10"
		6	210	0.75		Slight

The results given in Table 69 are deserving of attention since they show that transfusion of even 2 c.c. of heart blood may prove fatal to the guinea-pig. The death is not due to the mere speed of the injection since it will be shown that the serum of such rabbits was actually toxic in dose of 1 c.c. (Nos. 3 and 3a). In other words, the blood in these animals possessed an inherent toxicity. On the assumption that the blood makes up 10% of the body weight, the animals carried, respectively, 66, 114, and 103 guinea-pig lethal doses, which corresponds for all three with 50 guinea-pig lethal doses per kilo, or with 10 such doses per 200 gm. of rabbit. It has been shown in Part V that the normal rabbit can tolerate the injection of 13.4 guinea-pig lethal doses per 200 gm., and further that the immunized cachectic rabbit may carry 240 of such lethal doses per 200 gm.

The toxicity of the heart blood in these instances cannot be ascribed to ex-vivo precoagulation changes, for these are excluded by the speed of the transfer, and especially by the fact that such heart blood defibrinated very slowly when beaten with a glass rod; the yield of fibrin was unusually small and even more striking was the observation that the relative amounts of corpuscles and serum, after centrifugation, were distinctly abnormal, the ratio being about 1:3. This was true whether the blood was drawn from the heart or from the carotid, as was done in the case of Rabbit 3. The blood was clearly in an altered state, which in some way is related to, and probably conditions, the toxicity observed.

It will be seen that the serum of Rabbit 3, tested within 15 minutes after the withdrawal of the blood from the heart, was toxic in dose of 1 c.c. (Nos. 3 and 3a). The serum of only 1 of the 3 rabbits was tested, but without doubt that from the others would have been equally toxic. Hence, here are 3 normal rabbits capable of yielding sera which are fatal in dose of 1 c.c. per 200 gm. of guinea-pig. Only one other instance of normal rabbit serum having this high degree of toxicity (0.5 per 100 gm.) has been reported. Mita and Ito⁴ found 1 of 16 rabbits tested, to yield a serum of this potency.

This condition in the normal rabbit serves to establish the important fact that blood changes accompanied by increased toxicity may occur in apparently healthy normal animals; and that this reaction may become accelerated or intensified by immunizing injections. In the former some unknown cause, corresponding to the immunizing injection,

⁴ Ztschr. f. Immunitätsf., 1913, 17, p. 586.

tions, serves to develop the anaphylatoxin. The natural tolerance of the rabbit toward the anaphylatoxic disturbance insures it a resistance to conditions which rapidly prove fatal to the nontolerant or susceptible guinea-pig. This explains, perhaps, why it is practically impossible to keep guinea-pigs for any length of time in cold basement rooms, tho rabbits seem to thrive there perfectly.

The acquired or inherent anaphylatoxin of the normal or treated rabbit must be identified with the precoagulation anaphylatoxin which forms in drawn blood; and also with the induced anaphylatoxin which develops in sera treated with alien substances. The three conditions express, in reality, one and the same thing — an intramolecular change in a very labile plasma constituent. When the inherent toxicity is high, there is no increase during the coagulation of such blood; when it is at a minimum, as when 10 c.c. are nonfatal, the precoagulation toxicity becomes marked. In other words, the matrix is available in the latter for transformation, while in the former it has already undergone change.

AGAR AND ENDOTOXIN

A brief mention can be made at this point of the classic work of Pfeiffer⁵ on cholera immunity. He found that the intravenous injection of 0.5 mg. of live cholera bacilli per 100 gm. of guinea-pig, caused death. The dose for the organism killed by chloroform treatment for 10 minutes was 0.75 mg. The symptoms following such injections into immunized, that is sensitized, guinea-pigs were those of typical acute anaphylactic shock, while those in normal animals were similar but slower, with characteristic fall in temperature. These effects, as is well known, were ascribed to the presence of an 'endotoxin.'

Friedberger and Mita⁶ found that the moist culture of *Vibrio metschnikovi*, killed by heating at 60 C. for 2 hours, was fatal in dose of 0.25 gm. per 100 gm. of guinea-pig. When sensitized by a previous injection, they were killed by a second injection of from one-fifth to one-tenth this amount. The moist culture on the basis of 75% water would correspond with 0.062 gm. of the dried organisms. Similarly, dry tubercle bacilli were fatal in dose of 0.02 gm. per 100 gm. of guinea-pig, when given as a first injection. For previously immunized, or sensitized, guinea-pigs, the lethal dose was 0.005 gm. per 100 gm., a fourfold increase in toxicity as regards the treated animal. Here

⁵ Ztschr. f. Hyg. u. Infektionskr., 1894, 16, p. 272.

⁶ Ztschr. f. Immunitätsf., 1911, 10, p. 467. Centralbl. f. Bakteriol., R., 1911, 50 (Beihft), p. 58; that is, Bericht üb. d. 5. Tagung d. Fr. Ver. f. Mikrobiol. in Dresden, 1911.

again, the effects were those of anaphylaxis, and were explained as due to the liberation of endotoxin.

Seitz⁷ tested the toxicity of 17 different organisms by injecting guinea-pigs with from 5 to 100 mg. of the live cultures. Occasionally he obtained acute fatal shocks, tho usually death was delayed for from 1 to 25 hours. The dysentery bacillus, for example, in dose of 20 mg. killed a 200-gm. guinea-pig in 2 minutes. He concluded that the intravenous injection of saprophytic, as well as of virulent, bacteria caused anaphylactic poisoning, and that death was not due to embolism.

Müller⁸ made similar observations with boiled cultures of 4 organisms. He noted that distinct anaphylactic effects, at times acute deaths, were produced in normal animals injected with such material, and that the sensitized animals on reinjection were more liable to acutely fatal shock. The lethal dose for the former, in which acute death resulted, ranged from 7 to 23 mg. of the dried organisms, presumably for guinea-pigs of 200 gm.

These observations are sufficient to show that the dead and the living cultures of various organisms produce essentially the same poisoning, and that the dosage, with the exception of cholera vibrio, is about the same. It has been shown in Table 62 that agar can produce a similar typical shock in dose of 0.94 mg. per 100 gm. of guinea-pig, and that many such deaths can be obtained with from 1 to 2 to 3 mg. It is a rather remarkable fact that a substance apparently as inert as agar should be almost as toxic as the cholera vibrio, and possibly 20 times as toxic as the tubercle bacillus, and 62 times as toxic as *Vibrio metschnikovi*. The effects produced by the agar are the same, and yet one cannot speak of an agar endotoxin. The same holds true for the intoxications caused by kaolin, and by silicic acid, already mentioned in connection with Table 62.

This fact goes to show that the so-called toxicity of the cholera vibrio, as well as that of the tubercle, typhoid, and dysentery bacilli, pneumococcus, trypanosomes, etc., is of the same order as that of agar. The substance of the invading organism is not a poison, neither is it broken up into a poison by enzymatic action, but the whole cell or its fragments, like agar, kaolin, peptone, etc., induce a change in the plasma which results in the production of anaphylatoxin. The more perfect the comminution of such matter, the more toxic does it seem to be.

⁷ Ztschr. f. Immunitätsf., 1911, 11, p. 588.

⁸ Ibid., 1912, 14, p. 426.

In other words, much of what in the past has been designated as endotoxin becomes a fiction and thereby loses its specificity. The pathogenic or nonpathogenic organism, dead or alive, as well as non-cellular substances (organic and inorganic), acting on serum in the test tube, or on the plasma in the living body, create a disturbance which finds its expression in anaphylatoxic poisoning. One general principle underlies a host of so-called intoxications.

SUMMARY

Agar injected into guinea-pigs may produce a typical anaphylactic shock and death with characteristic autopsy findings.

With agar in the proper physical state this result can be produced by as little as 9.4 mg. of agar per kilo of body weight. This is less than the hitherto observed lethal doses of kaolin or silica, and less than those of most bacteria.

Agar sol, at 50 C., in dose of 10 c.c. (50 mg. of agar) usually is without effect, but it may produce typical shock and death.

Coarse suspensions of gel in salt solution yield atypical shock and findings.

Sol-gel mixtures with distilled water tend to yield an atypical shock; made with salt solution, they produce a typical anaphylactic shock, which is preceded by a definite period of incubation.

The rabbit appears to be somewhat less susceptible than the guinea-pig; 16.6 mg. per kilo caused death.

The rat is still less reactive to agar; it may tolerate from 30 to 50 mg. per kilo, but fatal shocks, preceded by a latent period, were obtained with 27, 32, and 80 mg. per kilo. This insusceptibility of the rat and of the rabbit parallels their behavior to injections of anaphylatoxin (Part V).

The in-vivo production of anaphylatoxin in agar-shocked rats and guinea-pigs (and possibly rabbits) is demonstrable by blood transfusion, and proof is thus given that the shock effects and death are due to this poison.

With a transfer time of 1 minute, the transfusion of 4 c.c. of normal rat blood resulted in typical fatal anaphylactic shock, due to the anaphylatoxin formed as the result of the precoagulation disturbance.

The transfusion method has shown that the blood of normal untreated rabbits may be toxic in dose of 2 c.c., and that the serum from such animals may be fatal in dose of 1 c.c. The apparently healthy rabbit may carry 50 guinea-pig lethal doses per kilo.

This inherent or acquired toxicity is to be correlated with that developed by immunizing injections, also with the precoagulation toxicity, and with that of normal serum, as well as with that induced in normal serum by alien substances. It will eventually be shown to be identical with that produced in specific anaphylactic shock.

A comparison of the toxicity of agar with the values given by Pfeiffer for the cholera endotoxin shows that the 'inert' agar is almost as active. Agar is many times more toxic than the ordinary pathogenic bacteria. The similarity in effects justifies the conclusion that much of the so-called 'endotoxin' of the various pathogenic organisms is of the same order as the toxicity of agar. In short, the common conception of endotoxin is fundamentally wrong.

The disturbance in the plasma caused by the introduction of alien substances results in the formation of anaphylatoxin the poisonous effects of which have been erroneously attributed to a liberation of the so-called endotoxin.

ANAPHYLATOXIN AND ANAPHYLAXIS

VII. PEPTONE ANAPHYLATOXIN

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SYNOPSIS

INTRODUCTION

INTRAVENOUS INJECTION OF PEPTONE INTO GUINEA-PIGS

THE LETHAL DOSE; VARIABLE RESISTANCE

SPEED OF INJECTION; IMMUNITY; ANTAGONISTIC SUBSTANCES

EFFECT ON BLOOD COAGULATION

INTRAVENOUS INJECTION OF PEPTONE INTO WHITE RATS

EFFECTS OBSERVED; THE LETHAL DOSE

INTRAVENOUS INJECTION OF PEPTONE INTO RABBITS

SYMPTOMS; THE LETHAL DOSE; IMMUNITY

EFFECT ON BLOOD COAGULATION

THE IN-VITRO PRODUCTION OF ANAPHYLATOXIN BY PEPTONE IN

RAT SERUM; IN GUINEA-PIG SERUM

THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN RATS; IN

GUINEA-PIGS; IN RABBITS

SUMMARY

The observations made in Ludwig's laboratory by Schmidt-Mülheim,¹ and by Fano,² that an injection of digestion products, that is, peptone, caused an intoxication characterized by a noncoagulable blood, served as the starting point of a long series of investigations. The early work was necessarily made with mixtures consisting of various hydrolytic products, and in view of the inherent difficulties it is not surprising, therefore, to find that very little was learned regarding the nature of the substance concerned, and much less regarding its mode of action. Even as late as 1900, Pick and Spiro³ resorted to the conception of a hypothetical substance or substances which they designated as "peptozyme," a term which represented no advance over the "peptotoxin" of Brieger.⁴ Although Witte's peptone, which consists largely of albumoses, was used frequently in such work, it was by no means certain that the action was due to these bodies. The studies of Chittenden, Mendel, and Henderson,⁵ of Underhill,⁶ and of

¹ Arch. f. Anat. u. Physiol., 1880, p. 33.

² Ibid., 1881, p. 277. Arch. ital. de biol., 1882, 2, p. 146.

³ Ztschr. f. physiol. Chem., 1900, 31, p. 272.

⁴ Ueber Ptomaine, 1885, 1, p. 17; Deutsch. med. Wchnschr., 1891, 17, pp. 821, 917.

⁵ Am. Jour. Physiol., 1899, 2, p. 142.

⁶ Ibid., 1903, 9, p. 345. Underhill and Hendrix, Jour. Biol. Chem., 1915, 22, p. 443.

others, however, served to show that certain proteoses were responsible for the observed effects.

The question of peptone-intoxication acquired a new interest with the discovery of anaphylaxis, for the reason that the symptoms and findings were essentially those of anaphylactic shock. The work of Vaughan⁷ directed attention to the protein-cleavage products as the probable poison in anaphylaxis, and about the same time, DeWaele⁸ pointed out the striking analogy between the two phenomena. Somewhat later, Biedl and Kraus⁹ emphasized the similarity of the two forms of poisoning in dogs, while Hirschfelder¹⁰ showed that the injection of Witte's peptone into guinea-pigs reproduced the picture of anaphylaxis. These facts obviously did not prove that anaphylactic shock was due to intoxication with peptone, but they at least served to show that the two had something in common.

If it was difficult to explain anaphylactic shock, it was equally difficult to account for the markedly poisonous property of proteoses. It was easy enough to assume that peptone was poisonous per se, or that on further cleavage it gave rise to the real poison. On the other hand it was conceivable that the proteose, strictly speaking, was not toxic but that it induced poison-production in the plasma of the animal. This view, rendered highly probable by the work on trypanosome and agar anaphylatoxin (Parts I to IV), was subjected to the test of experiment with the result that anaphylatoxin-production was shown to occur. This poison is formed in vitro when the peptone and serum are mixed; it is also produced in vivo when the peptone is injected into animals. Peptone, agar, and cellular matter (trypanosomes, bacteria, etc.) have, therefore, a common mode of action.

INJECTION OF PEPTONE INTO GUINEA-PIGS

At the outset, it was desirable to ascertain the effects of Witte's peptone on guinea-pigs, notwithstanding the fact that a number of investigators had concerned themselves with this subject.

Thus, Persano¹¹ found that the injection of more than 0.6 gm. per kilo of a commercial preparation caused rapid death, while DeWaele⁸ obtained some effect by intraperitoneal injections of Witte's peptone, as did also Pfeiffer and Mita.¹² Hirschfelder¹⁰ like Persano found that the intravenous injection of this

⁷ Jour. Infect. Dis., 1907, 4, p. 504.

⁸ Bull. de l'Acad. roy. de med. de Belgique, 1907, 21, p. 715.

⁹ Wien. klin. Wchnschr., 1909, 22, p. 363. Ztschr. f. Immunitätsf., 1910, 7, p. 222.

¹⁰ Jour. Exper. Med., 1910, 12, p. 586.

¹¹ Arch. ital. de biol., 1902, 37, p. 409.

product caused acute death and in addition, he noted the similarity to that of anaphylaxis; the dose employed by him was 0.98 to 2.8 gm. per kilo. Biedl and Kraus,¹³ using a dose of 1 to 1.2 gm. per kilo, obtained similar results.

Kumagai¹⁴ established 1.5 c.c. of 10% Witte's peptone as a single lethal dose per 200 gm. (0.75 gm. per kilo), while Ritz¹⁵ employing a 12% solution found the lowest dose to be 1.8 c.c. (0.87 to 0.93 gm. per kilo). It will be shown presently that an average lethal dose, one which kills about 50% of the test animals, may be as low as 0.6 c.c. of the 10% solution (0.3 gm. per kilo), an amount which is appreciably less than hitherto reported.

For the preliminary tests given in Table 70, a 10% solution of Witte's peptone was used; this was prepared by dissolving 10 gm. in 100 c.c. of salt solution (0.85%) at 60 C. The resulting suspension can be used as such, but for most of the work it was autoclaved at 110 C. for 15 minutes and then either filtered or centrifugated.

In Exper. A, the cloudy suspension, obtained by warming at 60 C., was used; for Exper. B, this suspension was centrifugated at 3000 revolutions for 4 minutes; for Exper. C, it was centrifugated at 8000 revolutions for 45 minutes; for Exper. D, it was heated to 110 C. for 15 minutes and filtered. The tests with these four solutions were made on the same day, while the others were made at different times. In Exper. E, the solution was autoclaved at 110 C. and then centrifugated at 8000 r. p. m. for 40 minutes. For Exper. F, the dry peptone was sterilized at 150 C. for 1 hour, then dissolved in sterile salt solution at 60 C. In Exper. G, the peptone was dissolved in distilled water at 60 C., and used as such, unfiltered, for Test 17, while for the two preceding tests it was first heated to 110 C. and filtered.

The symptoms and findings in guinea-pigs injected with peptone were the same as those produced by anaphylatoxin, by agar, or in specific anaphylactic shock. In addition to the usual effects—dyspnea, spasms, convulsions, and urination—a marked exophthalmos was noted. This symptom has seemingly been overlooked by previous observers. It is a striking feature when large guinea-pigs are used, and especially is it in evidence in rabbits injected with peptone. If recovery occurs, it disappears in a few minutes. The necropsy findings were those of typical anaphylactic shock—maximal distention of lungs, heart beating, absence of clot, blood fluid, and low blood pressure, as indicated by the difficulty of drawing blood from the heart for transfusion and other tests.

¹² Ztschr. f. Immunitätsf., 1909, 4, p. 439.

¹³ Zentrabl. f. Physiol., 1910, 24, p. 258. Centralbl. f. Bakteriöl., R., 1910, 47, Beiheft, p. 35.

¹⁴ Ztschr. f. Immunitätsf., 1913, 17, pp. 626, 636.

¹⁵ Ibid., 1911, 12, p. 654.

The Lethal Dose.—An inspection of the table will show that the lowest fatal dose was obtained in Expts. A and F, in which the unfiltered suspension, made at 60 C., was used. Seemingly, short centrifugation at 3000 r. p. m. decreased the toxicity (B); this was lowered still more by prolonged centrifugation at 8000 r. p. m. (C), or by autoclaving and filtering (D). In other words, the clear solution was less toxic than the cloudy suspension, the lethal dose of the former being 1.5 c.c. or more (C, D, E), while that of the latter was 0.75 to 1 c.c. (F, A). These figures, however, do not represent the minimal fatal dose, for it will be shown that smaller amounts may prove fatal.

TABLE 70
INJECTION OF 10% PEPTONE SOLUTION INTO GUINEA-PIGS

Expt.	Guinea-Pig		Peptone Solution*		Result
	Number	Weight	c.c.	gm. per kilo	
A	1	210	1.5	0.71	3/40". Typical shock
	2	220	1.0	0.45	3/40". " "
	3	220	0.5		Very slight
B	4	207	1.5	0.72	3/40". Typical shock
	5	185	1.0		Slight
C	6	215	4.0	1.8	3'. Atypical shock
	7	250	2.0	0.8	3'. Typical shock
	8	225	1.5		Very slight
D	9	210	2.0	0.95	3/10". Typical shock
	10	212	1.5		Severe
E	11	198	1.5	0.75	4/25". Typical shock
	12	170	"	0.88	6/10". " "
F	13	195	0.75	0.38	6'. Atypical shock
	14	198	0.5		Slight
G	15	185	4.0	2.1	2/20". Typical shock
	16	235	2.5	1.06	3'. " "
	17	206	1.5	0.72	3/40". " "

* Intravenously injected.

Thus, it will be seen from Tables 71 and 72 that 1 c.c. of the clear peptone solution may kill in about one-half of the tests (= 0.5 gm. per kilo), and furthermore (Table 75), even 0.6 c.c. (0.3 gm. per kilo) will give a like result. On the other hand, very exceptionally, recovery has been observed after a rapid injection (5 to 7 seconds) of 1 gm. per kilo. These facts indicate that the guinea-pig shows considerable variation as to its susceptibility to this intoxication. Moreover, it is to be noted that peptone is considerably less toxic than agar, which, as shown in Part VI, may kill in dose of 10 mg. per kilo.

Variable Resistance.—From what has just been said it is evident that no two guinea-pigs will necessarily show the same behavior to a given dose of peptone. As was the case with anaphylatoxic serum, individual variation in susceptibility is a striking feature which extends not only to the symptom complex as a whole, but also to the individual features thereof. Table 71, for example, presents the results of a series of tests made at intervals of 15 minutes, the peptone being kept at 37 C. Although the injection time was the same (45 seconds) and the dose employed was but 1 c.c., 4 tests proved fatal, whereas the other 6 showed slight or practically no effect. This variation in response may extend to the individual symptoms or changes. Thus, given the same injection of peptone into a series of guinea-pigs, the speed of coagulation of the blood when transferred to the test tube will vary considerably. Similarly, the drop in blood pressure is subject to much variation.

TABLE 71
INJECTION OF 10% PEPTONE, INCUBATED AT 37 C., INTO GUINEA-PIGS

Guinea-Pig		Intravenous Injection (c.c.)	Peptone Incuba- tion at 37 C. (hr.)	Result
Number	Weight			
1	203	1.0	—	Very slight
2	192	"	1/4	11'35"
3	191	"	1/2	2'55"
4	198	"	3/4	Very slight
5	212	"	1	Slight
6	195	"	1 1/4	Very slight
7	195	"	1 1/2	3'15"
8	203	"	2	Fair
9	185	"	2 1/4	Slight
10	195	"	2 1/4	Slight

The lethal dose is 0.49 to 0.52 gm. per kilo of guinea-pig.

Speed of Injection.—Table 72 presents another instance of variation, the experiment being similar to that given in Table 45. The animals were tested in pairs, the second one of each pair being injected within 30 seconds of the first, and the entire set of 6 pairs being injected in less than 45 minutes and in the order given. The immediate object of the experiment was to ascertain the effect of the speed of injection, and with that in mind the first 3 pairs were injected slowly, the injection being interrupted and 0.25 c.c. being given every 15 seconds; the total time of the injection was 45 seconds. The second 3 pairs were injected very rapidly, that is, in from 2 to 3 seconds. The same number of acute deaths was obtained in the first as in the second set, but the latter, in addition, developed 2 slow deaths.

The experiment shows that a very rapid injection is somewhat more injurious than one made slowly, a fact which has been recognized, concerning the dog, from the time of Schmidt-Mülheim and Fano. With an injection time of 2 minutes, a dose of 1.5 c.c. of peptone, even if diluted with salt solution or with distilled water, can be given quite safely, but the same dose injected in half that time is usually fatal.

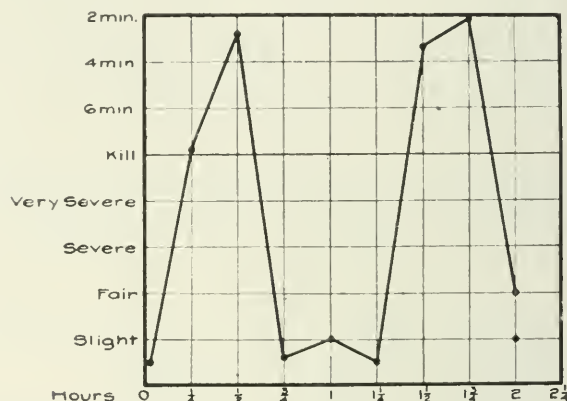


Chart 10. Apparent variation in the toxicity of a 10% solution of Witte's peptone, kept at 37 C. (Table 71).

TABLE 72

APPARENT VARIATION IN THE TOXICITY OF PEPTONE. INDIVIDUAL RESISTANCE.
SPEED OF INJECTION *

Exper.	Guinea-Pig		Peptone† (c.c.)	Result	Exper.	Guinea-Pig		Peptone† (c.c.)	Result
	No.	Weight				No.	Weight		
A	1	187	1.0		B	4	200	1.0	10 hr.
	1a	185	"	2'40" Slight		4a	200	"	7 "
	2	202	"	Moderate		5	196	"	3'50"
	2a	205	"	2'47"		5a	195	"	Slight
	3	205	"	Slight		6	203	"	3'52"
	3a	204	"	3'17"		6a	204	"	3'40"

* Nos. 1 to 3a given interrupted slow injection; Nos. 4 to 6a given very rapid injection.
† Intravenously injected.

Immunity.—Another well-known fact regarding peptone is that a certain immunity or tolerance is established by a nonfatal injection. In dogs, this immunity is seen in the behavior of the blood pressure, and of the blood itself which does not become incoagulable as it did after the first injection. In treated guinea-pigs, the immunity is evidenced by the fact that the second injection of an otherwise surely fatal dose

is tolerated with little or no effect. Thus, No. 1a of Table 72 when injected 3 hours later with 1.5 c.c. of a 10% peptone showed some slight respiratory disturbance and urinated, but developed no spasms, and when bled an hour later, the blood coagulated in the tube in 5 minutes.

Numbers 2 and 3 of Table 72 were likewise reinjected with 1.5 c.c. of the peptone, 3 hours after the first injection. Both showed practically no effect and consequently, half an hour later, they were given a third injection of a like amount, with no effect other than depression and some uneasiness. Twenty minutes later, they were given a fourth injection as before; No. 2 showed no effect, and 15 minutes later it was bled from the heart to ascertain the effect on the blood; this coagulated in the tube in 6 minutes. No. 3, however, after the fourth injection, developed slight jerky spasms which kept up for half an hour. An hour and a half after the injection it was very weak and on its side, and clearly was about to die. It was accordingly bled from the heart, and the blood was found to clot in the tube in 3 minutes.

By contrast with the foregoing, No. 5a, which also was reinjected with 1.5 c.c. of the peptone solution, 3 hours after the first treatment, responded with a severe typical shock and died in 6 minutes; the blood drawn from the heart 3 minutes after death clotted in the tube in 2 minutes. This does not mean that the blood was more coagulable than in the preceding instances, for if the coagulation time is reckoned from the moment of death, it would be 5 minutes. This result shows that in some individuals the immunity is either very fugitive or very fragile.

The repeated injection of a sublethal dose of peptone (0.5 c.c.), at intervals of 15 minutes, was also tried, with the object of learning the effect of such injections on the coagulability of the blood. It was rather unexpected to find that 1 guinea-pig died in 8 minutes after the second injection; the blood drawn from the heart 5 minutes after death did not clot in the tube until 7 minutes later, or 12 minutes after death. In another test, the guinea-pig showed no effect from the first and second injections, but died in about 15 minutes after the third; its blood coagulated in the tube in 3 minutes. Apparently, the injection of sublethal doses at short intervals is not tolerated as well as that of larger doses at longer intervals.

The immunity as regards a given dose of peptone (1.5 c.c.) can often be broken down by diluting this dose with 6 to 9 parts of salt

solution or distilled water. Nos. 1, 2, 4, and 5 of Table 78 present instances of this kind. This result, however, is not constant, especially if the first dose had been similarly diluted.

Antagonistic Action of Salt Solution.—Ritz,¹⁵ in testing the protective action of salt solution against peptone intoxication obtained somewhat irregular results, which in the main, however, showed that salt could protect. The variable results, without doubt, were due in part to the individual resistance of the animals, and, in part, to the delayed injections. It was desirable to make independent tests of the behavior of salt, especially for comparison with similar experiments with sodium carbonate.

A 10% solution of Witte's peptone (10 gm. + 100 c.c. of 0.85% NaCl) was used for the tests given in Tables 73 and 74. A so-called 30% solution of salt was prepared by dissolving 7.5 gm. of Kahlbaum's sodium chlorid in 25 c.c. of distilled water. The injections were made, as usual, into the jugular vein. As Ritz and others have noted, the injection of the concentrated salt solution must be made slowly to avoid a fatal shock. A careful watch of the respiration of the guinea-pig during the injection is desirable. The irritant action of the salt is usually seen in the production of tremors involving the abdominal muscles. The time required for the injection of 1 c.c. of the salt solution in the following tests ranged from $1\frac{1}{2}$ to $1\frac{3}{4}$ minutes. Immediately after the injection of the salt, the syringe was disconnected from the needle, which remained in the vein; a syringe with peptone was then attached and its contents injected, the time for the latter injection being 5 to 8 seconds. The total time from the beginning to the end of this double injection was $1\frac{3}{4}$ to 2 minutes.

From Exper. A, Table 73, it will be seen that 1 c.c. of the salt solution protected perfectly, in 3 of 4 tests, against 1.5 c.c. of the peptone solution, a dose which is almost invariably fatal. This amount of salt, however, did not protect against 2 c.c. of the peptone solution (Exper. B), corresponding in this respect to the like tests with sodium carbonate (Table 74, Exper. B). In the work with the latter, however, it was found that the alkali did protect against this amount of peptone, provided it was injected immediately before and after the peptone. Similar tests with salt (Exper. C) failed to give protection. The procedure followed in Exper. C was first to inject 0.5 c.c. of the salt solution, then the peptone which was followed, at once, by another 0.5 c.c. of salt. The total time from the beginning to the end of these injections (Nos. 9 and 10) was $1\frac{3}{4}$ and $2\frac{1}{4}$ minutes, respectively.

TABLE 73
PROTECTIVE ACTION OF SALT AGAINST PEPTONE

Exper.	Guinea-Pig		c.c. 30% NaCl	c.c. 10% Peptone	Result
	Number	Weight			
Control	1	193	—	1.5	4'15". Typical shock
	2	182	—	"	13'20"
A	3	185	1.0	1.5	Practically nil
	4	190	"	"	5'. Typical shock
	5	193	"	"	Like No. 3
	6	206	"	"	" " "
B	7	186	1.0	2.0	2 hr. 6 min.
	8	191	"	"	5'25". Typical shock
C	9	191	1.0*	2.0	5 hr.
	10	190	"	"	3'45"

* Half before and half after peptone injection.

Antagonistic Action of Sodium Carbonate.—It was pointed out in Part III that Friedberger and Moreschi had observed that anaphylatoxin was destroyed by treatment with normal NaOH, and it was shown, further, that a triple alkalized serum did not give rise to the poison on subsequent treatment with agar (Table 36). Other tests, as yet not described, have demonstrated that anaphylatoxin is destroyed on contact with sodium carbonate, and these facts suggested that a similar reaction could take place in vivo, and that protection could thus be obtained against peptone and even against specific anaphylactic shock. The experiments given in Table 74 show that sodium carbonate actually does protect against peptone intoxication.

The method of procedure was the same as that given above in connection with the tests with salt. A normal solution of sodium carbonate was employed, and, as this was tolerated much better than the more concentrated salt solution, the injection time was reduced to 1 minute. The injection time for the peptone ranged from 8 to 20 seconds, and the total time from the beginning to the end of the double injection was 1¼ to 1¾ minutes.

It will be seen from Exper. A that the alkali protected against an otherwise fatal dose of peptone, the result corresponding to that of the like test, Exper. A of Table 73 where concentrated salt solution was used. The same amount of alkali, given in a single dose, did not protect against 2 c.c. of the peptone (Exper. B), but given in 2 injections, 0.5 c.c. before and 0.5 c.c. immediately after the peptone, it did protect. (Exper. C). In the last test, No. 9, the alkali was given in 2 injec-

tions, each of 1 c.c., with the result that death occurred. An excess of alkali (2 c.c. or more) injected alone, can produce an acute death. Autopsy will disclose findings like those of anaphylatoxic poisoning.

On comparing the results given in Tables 73 and 74, it will be noted that normal Na_2CO_3 (5.3%), in about one-sixth the concentration of the salt, is as active and even more so than the latter. In view of the fact that peptone intoxication is due to the in-vivo production of anaphylatoxin, it is significant that the action of the latter can be completely neutralized or destroyed by the administration of a small amount of alkali; the presence of the latter may even prevent the disturbance which leads to poison-production. While this is not the place to consider the fundamental conception regarding anaphylatoxin-production, it may be well to state the belief that the clinical application of this principle is of far-reaching importance; applied in 3 very severe cases (salvarsan poisoning, Landry's ascending paralysis, and multiple exudative erythema) it has yielded already surprising results.

TABLE 74
PROTECTIVE ACTION OF NORMAL SODIUM CARBONATE AGAINST PEPTONE

Exper.	Guinea-Pig		c.c. Normal Na_2CO_3	c.c. 10% Peptone	Result
	Number	Weight			
Control	1	192	—	1.5	7'20"
	2	192	—	"	5'40"
A	3	190	1.0	1.5	Practically nil
	4	200	"	"	" "
B	5	200	1.0	2.0	3'30"
	6	198	"	"	3'55"
C	7	185	1.0*	2.0	Practically nil
	8	184	"	"	" "
	9	185	2.0*	"	5'10"

* Half before and half after peptone injection.

Antagonistic Action of Normal Serum.—It is well known that the addition of normal serum to an organ extract renders the latter less toxic. A similar observation concerning kaolin anaphylatoxin was made by Mutermilch,¹⁶ who found that the toxic property disappeared on treatment with either fresh serum or extracts of organs of guinea-pigs. His interpretation of a limited number of experiments may be questioned in view of the fact that guinea-pigs vary considerably in resistance (Table 45). Be that as it may, the belief that serum is antag-

¹⁶ Ann. de l'Inst. Pasteur, 1913, 27, p. 90.

onistic to organ extracts is generally held, and it would be in order to expect a like behavior concerning peptone. In an extensive series of tests, having for their object the demonstration of anaphylatoxin-production in vitro, it was repeatedly observed that a serum-peptone mixture could be relatively inert, though the amount of peptone present, were it given by itself, would yield a large percentage of deaths. Thus, it will be shown that serum mixtures containing 0.5 or 0.3 c.c. of 20% peptone are quite nontoxic, though, as demonstrated in Tables 71 and 72, a dose of 1 c.c. of a 10% solution of peptone was fatal in about one-half of the tests; the control tests, Nos. 6 to 7a of Table 75, gave a similar result with 0.3 c.c. of a 20% solution of peptone.

TABLE 75

ATTEMPT AT ANAPHYLATOXIN-PRODUCTION. PROTECTIVE ACTION OF GUINEA-PIG SERUM AGAINST PEPTONE. THE DOSE REPRESENTS 3 C.C. SERUM AND 0.3 C.C. OF 20% PEPTONE

Exper. and Ratio	Guinea-Pig		Mixture			Result
	No.	Weight	Incubation at 0 C. (min.)	Incubation at 38 C. (min.)	Intra- venous Injection	
Control A 1:50	1	184	2	—	3.3	Nil
	1a	186	"	—	"	"
	2	190	6	—	"	"
	2a	191	"	—	"	Very slight
B 1:50	3	193	9	10	3.3	7/43"
	3a	193	"	"	"	Very slight
	4	205	"	15	"	"
	4a	200	"	"	"	Moderate
	5	196	"	25	"	Very slight
	5a	197	"	"	"	45"
Control C	6	205	—	—	3.3*	4/20"
	6a	200	—	—	"	Slight
	7	190	—	—	"	Very slight
	7a	190	—	—	"	5/50"

* The control C received a mixture of 3 c.c. of salt solution + 0.3 c.c. of 20% peptone.

For the experiments given in Tables 75 and 76, made on the same day, a 20% solution of Witte's peptone was prepared by adding 20 gm. of the latter to 100 c.c. of 0.85% salt solution; the mixture was heated on the water bath at 100 C. for 15 minutes and then filtered. For the tests given in Table 75, a single pool of 30 c.c. of fresh guinea-pig serum was used. To this serum, previously iced, 3 c.c. of the peptone

solution were added, and the mixture, after being swung for half a minute, was placed in cracked ice. The injections were made in pairs, the first being within 2 minutes and the second within 6 minutes after preparing the mixture; these first 4 tests served as preliminary controls. When injections were made in pairs, the second animal received its dose within half a minute of the first.

After Tests 1 to 2a had been made and the mixture kept at 0 C. for 9 minutes, it was transferred to the Roux water bath at 38 C., and further duplicate tests were made after 10, 15, and 25 minutes, with

TABLE 76

ATTEMPT AT ANAPHYLATOXIN-PRODUCTION. PROTECTIVE ACTION OF GUINEA-PIG SERUM AGAINST PEPTONE. THE DOSE REPRESENTS 3 C.C. OF SERUM AND 0.5 C.C. OF 20% PEPTONE

Expt. and Ratio	Guinea-Pig		Mixture			Result
	No.	Weight	Incubation at 0 C. (min.)	Incubation at 38 C. (min.)	Intra- venous Injection	
A 1:30	1	200	16	—	3.5	Very severe shock
	2	197	18	—	"	Slight
	3	201	20	—	"	Very slight
	4	195	23	—	"	" "
B 1:30	5	200	—	10	3.5	Very slight
	5a	200	—	"	"	Severe
	6	200	—	20	"	Very slight
	6a	200	—	"	"	" "
	7	210	—	30	"	" "
	7a	208	—	"	"	" "
	8	190	—	50	"	" "
	8a	195	—	"	"	" "

For control tests as to the action of corresponding amount of peptone, see Tables 71 and 72.

the expectation of finding an increased toxicity due to anaphylatoxin-production. The first test (No. 3) gave the desired result, but the companion test (No. 3a) and the subsequent trials were not as favorable. The apparent flare-up in toxicity might be ascribed as due to a very susceptible guinea-pig (No. 3); it is conceivable, however, that the anaphylatoxin-production was started, then checked and destroyed by the excess of the serum, and more especially by its alkaline content. Whether this be true or not, the striking fact remains that of 10 tests only 1 acute and 1 subacute death were obtained, whereas of the 4 control tests (Nos. 6 to 7a), which received the same amount of peptone diluted with salt solution, two acutely fatal shocks resulted.

It would appear from the series of tests just given that the toxic action of peptone is counteracted by normal guinea-pig serum. This conclusion is fully corroborated by the two series of tests given in Table 76 which were made with another pooled serum (50 c.c.) used about 3 hours after bleeding. The same peptone solution was used as that of the preceding series of tests (Table 75).

In Exper. A, which was of a preliminary character, the mixture consisting of 2 c.c. of the 20% peptone and 12 c.c. of the pooled serum was placed in cracked ice and tests (Nos. 1 to 4) were made at intervals as indicated; these tests clearly show that the toxicity of the peptone, in the dose employed, namely, 0.5 c.c. 20% solution, was decreased by admixture with serum at 0 C. This dose of peptone is the equivalent of 1 c.c. of 10% solution, which, as shown in Tables 71 and 72, is fatal in about one-half of the tests. This result was confirmed by the series of tests in Exper. B, where a mixture of 5.5 c.c. of the 20% peptone and 33 c.c. of the same pooled serum was placed at once at 38 C., the tests in this case being made in pairs. The entire series of tests (Nos. 1 to 8a), it may be added, were made within 70 minutes. It will be noted that in Exper. B there is practically no evidence of toxicity, though the dose of peptone present in the serum mixture, were it given by itself, would have produced fatal results in one-half the tests. The injection time in all of these tests, it may be added, was but 5 to 10 seconds.

These experiments clearly show that the peptone toxicity is suppressed, regardless of the question as to whether such toxicity is due directly to the peptone, or to anaphylatoxin induced by its presence. As mentioned, it is possible that this antagonistic action of the serum is, in part at least, due to the alkali content of the serum. Given a pool of serum with a relatively high alkali content, actual or potential, it is to be expected that it would be less easily toxified by peptone; or, in other words, it would show a greater antagonistic action than one having a relatively lower alkali content. Or, stated in another way, a serum having a low primary toxicity may be expected to show a greater neutralizing power concerning peptone than one possessing a high primary toxicity.

The neutralizing action of serum, as shown toward peptone, may furnish a basis for the rather empirical practice of injecting serum, or of transfusing either normal blood or that of alkalinized persons.

Coagulation of Blood in Guinea-Pigs Injected with Peptone.—The

fact that various substances when injected intravenously into dogs render the blood noncoagulable is well established. The first observation of this kind was made by Albertoni,¹⁷ who found that the injection of hydrochloric acid and pepsin, or of pancreatin, produced this effect. Schmidt-Mülheim followed with the discovery of the action of peptone, and this was promptly confirmed by Albertoni and by Fano. A little later, Salvioli¹⁸ observed a similar effect in dogs when injected with diastatic enzymes of plant or animal origin, including whole saliva; he noted, however, that twice the dose given dogs had no effect in the treatment of rabbits or guinea-pigs, either upon the coagulability or upon blood pressure. Persano,¹¹ as mentioned heretofore, found that the injection of a peptone caused rapid death in guinea-pigs and that such injection had no anticoagulant effect; in fasting guinea-pigs, he found that a nonfatal dose gave a retard of 3 to 5 minutes, provided the blood was drawn from 2 to 5 minutes after the injection. Neither Hirschfelder, Biedl and Kraus, Ritz, or Kumagai made any special observations as to the state of the blood other than that, after death, the blood in the heart was fluid.

As mentioned heretofore, the heart of guinea-pigs which die from peptone injection is free from clot and the blood is fluid when the examination is made a few minutes after death. While no special effort was made to determine how long this fluidity would persist, the records show a score of observations in which the blood within the heart was perfectly fluid for 6 to 19 minutes after death. How much longer the heart would have remained free from clot cannot be stated. The fact remains that a peptone shock leaves the blood in the heart in a less coagulable state.

In view of the fact that the dog's blood after peptone injection remains perfectly fluid in the test tube, it seemed as if under proper conditions a similar result should be obtainable with the guinea-pig. This was all the more to be expected since retarded coagulation, and even complete incoagulability had been obtained with the blood of guinea-pigs injected with anaphylatoxin (Part V), and the latter condition had also been successfully procured in specific anaphylactic shock (Part IX). Considerable effort was therefore made to secure like results with guinea-pigs after peptone injections, but the outcome, tho fair, was by no means entirely satisfactory, since delayed coagulation but not strictly incoagulable blood was obtained. Perhaps this is

¹⁷ Centralbl. f. d. med. Wissensch., 1878, 16, p. 641; 1879, 17, p. 596; 1880, 18, p. 577.

¹⁸ Ibid., 1885, 23, p. 913.

to be expected in the light of results to be presented which indicate that, concerning the guinea-pig and rabbit, the peptone is a relatively weak, disturbing agent.

In order to test the coagulability of the blood after peptone injection, the heart was exposed either before, or just at death, or at a definite time after death, and the blood was quickly drawn up into a plain heart pipet (Fig. 1), the tip of which was then sealed with wax. Obviously, the glass rod, ordinarily present in the pipet, was omitted. Observations were made every minute and the time of beginning and that of completed coagulation were noted. The tables indicate the injection time as well as the time which elapsed from the end of the injection to the end of the bleeding operation. The actual time required for drawing the blood varied usually from 5 to 10 seconds, but when drawn after death, on account of the fall in blood pressure, this time was somewhat increased. The amount of blood thus drawn varied from 2 to 5 c.c. The index of death was the last nasal twitch.

The alteration in coagulability of guinea-pig blood is subject to individual peculiarities of the species, and is not entirely conditioned by the amount of the peptone, or by the speed of injection, or by the time of withdrawal. This change in the blood, like that of pressure and respiration, etc., is influenced by factors inherent or developed within a given individual. It is well known that dogs show similar variations, and this will also be found to be the case with rabbits. It may be added that Camus⁴⁹ found that injections of milk into dogs likewise gave extreme variations as to coagulability. As a rule, dogs respond readily to peptone injection with an incoagulable blood, whereas guinea-pigs, it will be seen, offer great resistance to this change.

A series of tests, made under varying conditions, are presented in Table 77. In Tests 2 to 5, inclusive, in which, according to plan, the heart blood was drawn exactly 3 minutes after the injection of the peptone, there were 2 marked delays in coagulation (Nos. 2 and 4), while 2 coagulated in about normal time. The blood of No. 2 remained perfectly fluid in the test tube for 15 minutes after withdrawal, but when reexamined 10 minutes later it had coagulated. The heart of this animal examined 16 minutes after the injection was found to be free from clot.

The first 12 tests given in Table 77 were made on one day. The blood was drawn at a definite time, as just stated, or at once after

⁴⁹ Compt. rend. Soc. de biol., 1900, 52, p. 787.

death, or at a fixed time thereafter, or before death as soon as the eye reflex had been lost. With the two exceptions noted, little effect was seen in regard to coagulation. The dose of peptone, which was the same in all twelve, represented a usually fatal amount. It was possible that a greater disturbance could be set up with a larger dose, and accordingly, on the following day, Tests 13 to 19, were made. In these the blood was drawn at or before death; of the 7 tests, 3 showed some delay, especially No. 15. It is possible that a better result would have been obtained with these increased doses had the blood been drawn 1 or 2 minutes after death. As the results stand, a moderate increase in the amount of peptone had no marked effect.

More striking changes, however, were obtained when a 30% solution of peptone was injected in doses corresponding to 3, 6, and 9 gm. per kilo (Nos. 20, 21, 22). These relatively large doses of peptone produced the most marked effect on the coagulation. Thus, in No. 22, the blood showed no sign of clotting until 22 minutes; at 35 minutes the clot was soft and floating and this incomplete clot persisted for 24 hours. In Test 21 the clot showed exactly the same behavior. Test 20, it will be seen, likewise showed a marked retardation, complete coagulation not taking place until 40 minutes.

It is evident from the results given in Tables 77 and 78 that, in so far as coagulation is concerned, the guinea-pig offers an extreme resistance to the action of peptone; this behavior is in striking contrast to that observed in dogs, where according to Thompson,²⁰ even as small an amount as 0.02 gm. per kilo may cause incoagulability. In guinea-pig 22, 450 times this amount of peptone produced but an incomplete coagulation. It is quite possible that if a larger number of tests, similar to Nos. 21 and 22 had been made, some would have shown complete incoagulability. Be that as it may, the fact remains that while peptone produces in guinea-pigs an acute fatal shock more readily than in dogs, the reverse is true in regard to the blood behavior. In the guinea-pig, peptone does not readily induce sufficient disturbance to give rise to much poison, or to a marked change in the blood. As pointed out in connection with the question of lethal dose, peptone is at best only one-thirtieth as toxic as an agar suspension.

It may be of interest to note that if a second portion of blood is drawn from the heart, it appears to coagulate more rapidly than the first. Indeed such blood may clot within 1 minute after removal. If,

²⁰ Jour. Physiol., 1896, 20, p. 455.

however, the time which has elapsed from the end of the injection to this second withdrawal is added to this coagulation time it will be found to correspond to that of the first portion. Thus, in Test 15, in which the coagulation time of the first portion was 16 minutes, a second portion drawn 16 minutes after the injection clotted in 2 minutes (+++). Again, in Test 17, in which coagulation occurred in 9 minutes, a second portion drawn 9 minutes after the injection clotted

TABLE 77

EFFECT OF PEPTONE ON COAGULATION OF BLOOD IN GUINEA-PIGS. NOS. 20 TO 22 RECEIVED A 30% SOLUTION; ALL OTHERS A 10% SOLUTION OF WITTE'S PEPTONE

Exper.	Guinea-Pig		Intra-venous Injection (c.c.)	Injection Time (sec.)	Interval from End of Injection to End of Bleeding	Time of Death	Clot Begins (+) (min.)*	Clot Complete (++++)(min.)
	No.	Weight						
A	1	192	1.5	4	2'33"	3'33"	(3)	4
	2	207	"	5	3'		(15)	25
	3	199	"	"	3' 5"		2	3
	4	204	"	"	3'		12	14
	5	211	"	"	3'	4'30"	2	3
	6	205	"	"	4'		(1)	2
	7	190	"	3	4' 7"	6'	2	3
	8	185	"	5	4' 5"	5'15"	(2)	3
	9	215	"	4	4'11"	5'	(3)	4
	10	209	"	5	5'	3'45"	(2)	3
	11	210	"	3	5'32"	3'40"	2	3
	12	197	"	5	3'20"	3'15"	3	6
B	13	195	2	5	2'40"	4'	(3)	4
	14	202	"	"	2'35"	2' 5"	5	6
	15	212	"	"	2'10"	3'15"	14	16
	16	195	3	10	1'50"	2'40"	(1)	2
	17	201	"	"	2'55"	3'20"	6	9+++
	18	193	"	5	2'50"	2'35"	(1)	2
	19	202	"	3	3' 5"	3'	(2)	2
C	20	197	2	8	3'37"	2'10"	7	40
	21	197	4	10	3'28"	2' 3"	18	40 ---+
	22	195	6	"	4'	1'30"	22	35 ---+

* The figure in parenthesis indicates that no coagulation had taken place at that time. The last nasal twitch, indicating death, was observed at times after the blood was drawn. In Nos. 21 and 22 the coagulation remained incomplete for 24 hours.

in 1 minute (+++). It is evident, therefore, that while the blood which remains in the heart is perfectly fluid, it is nevertheless undergoing a change parallel to that in the first tube, and that withdrawal of such blood, with the added disturbance due to glass contact, suddenly forces the reaction to completion.

In the tests given in Table 77, the peptone solution was injected in the original concentration. In view of the fact that an injection of

diluted agar (Table 61) gave an incoagulable blood, it seemed that possibly similar results could be obtained, if the action of the peptone was assisted, as it were, by the addition of distilled water. With this object in view, a series of tests were made in which the peptone was diluted with 4 to 9 parts of salt solution or with distilled water. The results are given in Table 78.

TABLE 78
EFFECT OF DILUTED PEPTONE (10%) ON COAGULATION OF BLOOD IN GUINEA-PIGS. DILUTIONS
MADE WITH DISTILLED WATER, EXCEPT IN NOS. 1 TO 3, IN WHICH
SALT SOLUTION WAS USED

Exper.	Guinea-Pig		Peptone	Injection Time (sec.)	Interval from End of Injection to End of Bleeding	Time of Death*	Clot Begins (+) (min.)	Clot Complete (++++) (min.)
	No.	Weight	Intravenous Injection (c.c.)					
A	1	183	15 (1.5 + 13.5)	80	4'40"	3'	6	7
	2	175	15 (" + ")	70	8'45"	6'50"	12	14
	3	175	15 (" + ")	68	16'30"	14'35"	(3)	4
B	4	185	10 (1 + 9)	60	3'25"	1'50"	7	8
	5	180	10 (" + ")	55	4'10"	2'30"	(4)	5
	6	203	10 (" + ")	60	4'	2'40"	7	10
	7	210	10 (" + ")	"	4'55"	3'20"	(2)	3
	8	200	10 (" + ")	"	15'40"	12'15"	(1)	2
	9	199	10 (" + ")	55	13'15"	11'10"	3	4
C	10	205	10 (1 + 9)	55	2'45"		(2)	3
	11	187	10 (1.5 + 8.5)	"	2'15"		(1)	2
	12	204	10 (" + ")	"	3'30"		(15)	20+
	13	204	10 (" + ")	"	4' 2"		3	4
	14	204	10 (" + ")	60	3' 2"		5	6
	15	205	10 (" + ")	90	13'50"	13'	(15)	28++++
D	16	213	10 (2 + 8)	55	2'50"	2'40"	2	3
	17	200	10 (" + ")	60	3'30"	2' 5"	3	5+++
	18	217	10 (" + ")	55	3'55"	2'35"	2	3
	19	210	15 (2 + 13)	65	3'45"	2'55"	(2)	3

* The time of death is the interval from end of injection to the last nasal twitch. In No. 15, the blood remained incompletely coagulated and soft for 45 minutes, after which it was not examined. In No. 12, the blood had a slight clot (+) and remained so for 2 hours.

An examination of this table will show that while the majority of tests show no marked retardation, a few gave evidence of delayed coagulation. One can safely designate as a delay any test in which the first sign of coagulation does not appear until 5 minutes or later. On this basis 7 of the 19 tests showed delay. The most marked reactions were noted in Tests 2, 12, and 15. In No. 12, there was no evidence of coagulation for 15 minutes; reexamined at 20 minutes it appeared

to have a slight deposit of fibrin on the glass, but there was no appreciable increase for 2 hours (+). This was the nearest approach to an incoagulable blood that was obtained. The heart of No. 12, when opened 19 minutes after the injection, showed no sign of clot. In No. 15, in which death was subacute, the blood was also perfectly fluid up to 15 minutes; reexamined at 28 minutes it had a soft floating clot (+++), which remained thus during the next 20 minutes, after which no further observations were made.

Incidentally, it may be mentioned that while 1 c.c. of the 10% peptone is fatal in about half of the tests (Tables 71 and 72), when this amount of peptone is diluted with 10 parts of distilled water, it appears to be more fatal (Nos. 4 to 9). It has already been pointed out that an injection of peptone which would confer immunity or tolerance to subsequent reinjection of 1.5 c.c. of the solution, does not protect, as a rule, against 1 c.c. which has been diluted with 9 parts of water. Exceptions, however, are to be found, and a previous dose of diluted peptone may protect against a second dose of 1.5 c.c. likewise diluted.

Another attempt at producing a more marked noncoagulation consisted in giving a preliminary injection of N/5 HCl followed at once by 4 c.c. of a 20% peptone. With 0.65 c.c. of the acid, coagulation occurred in 4 minutes; with 1.4 c.c. of the acid, the first sign (+) was obtained at 8 minutes and the coagulation was complete at 10 minutes. With 2 c.c. of acid, the blood coagulated in 5 minutes. It may be added that with an injection time of 1 minute, even 7 c.c. of the acid could be given without any effect; 10 c.c. caused death in about 12 minutes; and 15 c.c. resulted in death in 65 seconds, with dyspnea and convulsions. Blood drawn from the heart, in this case, 3 minutes after death remained entirely free from clot for 33 minutes, after which no further examination was made.

A few attempts were made to favor the action of the peptone by adding 0.1 c.c. normal sodium carbonate to 0.5 or 1 c.c. of the 10% peptone. In these tests, the blood coagulated in from 3 to 5 minutes. A larger dose of alkali may have given a better result, but it is to be remembered that an excess of alkali may counteract the toxic effect of peptone (Table 74).

INJECTION OF PEPTONE INTO WHITE RATS

Apparently, the rat has not been used hitherto in work with peptone. The white mouse has been tested by Ritz and Sachs,²¹ who found that peptone was toxic for this species while anaphylatoxin produced no severe effects and never death; since mice may die of specific anaphylactic shock they concluded that evidently more poison was produced in-vivo than was injected with the anaphylatoxic serum.

It was particularly desirable to ascertain the effects of injections of Witte's peptone into white rats, in view of the results which had been obtained with agar (Table 64). It was somewhat surprising to find that such injections produced severe intoxication and even death, more readily than was the case with agar, but a consideration of the dosage employed soon showed that peptone, in reality, was the more feeble of the two. The symptoms and findings were those of anaphylactic poisoning and, as will be shown, they are due not to peptone directly, but to changes induced within the rat.

For the tests given in Table 79, a 10% solution of the peptone in 0.85% salt solution was used. This was sterilized at 110 C. and then

TABLE 79
INJECTION OF 10% PEPTONE SOLUTION INTO WHITE RATS

Exper.	Rat		Peptone Solution*		Result
	No.	Weight	c.c.	gm. per kilo	
A	1	120	2.0	1.6	At once on side; dyspnea
	2	105	"	1.9	Dyspnea; depressed
	3	145	2.5	1.7	Moderate dyspnea
	4	135	3.0	2.2	" " ; slight spasm
	5	110	"	2.7	4/30". Typical shock and autopsy
	6	150	5.0	2.3	5". " " " "
B	7	148	3.0	2.0	At first nil, then severe shock
	8	200	4.0	2.0	2/31". Typical shock and autopsy
	9	145	3.7	2.5	2S". Typical shock

* Intravenously injected.

centrifugated. The injections were made intravenously and as a rule into the femoral vein, which is incomparably better adapted for the purpose than that of the tail. The injection time was not noted in the early tests, but in those made later it ranged from 10 to 30 seconds. The two groups of tests were made on different days and with different peptone solutions.

²¹ Centralbl. f. Bakteriöl., R, 1911, 50, Beiheft, p. 45.

The results obtained were very striking. Thus, while 1.5 c.c. of the peptone solution appeared to give little or no effect, a dose of 2 to 2.5 c.c. developed more or less dyspnea from which the rat soon recovered. A dose of 3 c.c. may be taken as the average lethal amount, since it proved fatal in 1 of 3 trials. Individual variation in the resistance of the rat occurs, the same as in other animals. In the fatal cases, after a slight delay, dyspnea rapidly developed together with severe spasms and convulsions; the animal was soon thrown and acute death resulted in 3 of the tests; in No. 9, the first shock caused a near-kill, but the respiration gradually returned; complete recovery, however, did not take place and a subacute death occurred.

The autopsy findings were perfectly typical of anaphylactic shock—maximal distention of lungs, heart beating, absence of clot, and blood perfectly fluid. The heart blood of No. 5, 5 minutes after death, was transferred to a small test tube and examined every minute; it began to clot 7 minutes later and was solid at 8 minutes, or 13 minutes after death. In the case of No. 8, the blood was drawn 10 minutes after death; 5 minutes later it was still fluid, but it had set (+++++) at 6 minutes, or 16 minutes after death. In another test, in which the rat (155 gm.) received an injection of 3 c.c. and was used for a transfusion experiment, the blood drawn 10 minutes after death showed the first indication of clot, 11 minutes later, and gave a complete clot at 12 minutes, or 22 minutes after death (Table 92, No. 3). These results clearly show that considerable retardation in clotting time occurs as the result of peptone injection into rats. Considering the number of tests made, they appear to be better than those given by the guinea-pig (Table 77).

The Lethal Dose.—Knowing the relative resistance of the rat to anaphylatoxin and to agar, it was to be expected that peptone would be less poisonous than it was for the guinea-pig. It will be seen from Table 79 that the least lethal dose per kilo of rat is 2 gm.; an amount which is about 7 times larger than that of the guinea-pig (0.3 gm. per kilo) (Table 75). It is clear, therefore, that the rat shows a greater resistance than does the guinea-pig to peptone, agar, anaphylatoxin, and specific shock.

Of further interest is the fact that peptone is much less toxic than agar. Thus, on reference to Table 64 it will be seen that the least lethal dose of agar per kilo of rat was 0.027 gm., whereas that of peptone, as shown, was 2 gm., a ratio of 1:74. In other words, in regard

to the rat, agar when in the proper state of division may be 74 times more toxic than Witte's peptone. A similar comparison of the two substances in regard to the guinea-pig shows that agar is at least 30 times more toxic than peptone (0.009 gm. vs. 0.3 gm. per kilo).

INJECTION OF PEPTONE INTO RABBITS

The rabbit has served for a number of peptone studies, largely because of its peculiar behavior to this substance. Fano noted that the rabbit, unlike the dog, did not respond to peptone injections, either with a drop in blood pressure or by a change in the coagulability of the blood. The amount used by him, however, was but 0.3 gm. per kilo, which is altogether too small for the rabbits. Albertoni (1880), a year before, had found that peptone was inactive in the treatment of the rabbit and sheep. These and other observations, based on insufficient data, led to the general belief that the rabbit was wholly refractory, or naturally immune to peptone. Incidentally, it may be well to recall that the rabbit is also looked upon as being relatively immune to specific anaphylactic shock.

The rabbit, however, is not immune either to peptone or to the anaphylactic shock. Grosjean²² was the first to show that rabbits did give some reaction with a propeptone (proteose) and a peptone which he prepared. He found that the injection of 0.3 gm. or less per kilo of his propeptone had no effect on clotting; the injection of 1.7 gm. per kilo likewise failed to show any effect on the coagulation, but in this experiment the blood was not drawn until 50 minutes after the injection. Had it been drawn shortly after injection, the result, without doubt, would have been different. This dose, however, caused a very rapid fall in blood pressure, which soon returned to the normal. The peptone in like dose gave a slight drop, followed by a rise in pressure which then gradually fell below normal. After the injection of this peptone, the blood drawn 80 minutes later coagulated normally, whereas, when drawn 15 minutes after the injection, it began to coagulate in 13 minutes, and the coagulation was complete in 45 and 60 minutes (2 tubes from 1 rabbit).

The work of Grosjean was extended a few years later by Gley,²³ who tested a propeptone prepared according to the method of the former. This product in dose of 1.5 gm. per kilo was always rapidly

²² *Mémoires couronnés et autres mémoires, l'Acad. roy. de Belgique*, 1892, 46, Mémoir 2, pp. 24, 25, 28. *Arch. de Biol.*, 1892, 12, p. 381.

²³ *Compt. rend. de la Soc. de biol.*, 1896, 48, p. 658.

fatal, a result, it may be added, not obtained by Grosjean. With the blood of 6 of 7 animals, thus tested, Gley obtained an appreciable decrease in coagulability. In 1 instance, the coagulation was retarded for 20 minutes, in 1, for 30 minutes, in 3 others, for more than an hour, and in 1, the blood remained fluid for 2 hours and then formed a soft clot. The extreme activity of his product almost raises the question whether all the ammonium sulfate used in the process had been removed. The animals injected showed a rapid fall in blood pressure, violent tetanic convulsions, the respiration stopped at once as did also the heart.

It may be added that Gley was unable to obtain incoagulable blood with Witte's peptone, and in a later paper²⁴ he apparently contradicted himself by stating that propeptone (presumably meaning Witte's peptone) exerted no influence upon the coagulability of blood of the rabbit. On the other hand, Nolf²⁵ using Witte's peptone, was able to get a fall in blood pressure, but while 0.03 gm. per kilo was active in the dog, even 1 gm. per kilo was not always active in the rabbit. Similarly, the rabbit is resistant to injections of milk, snail's blood (Camus), strawberry extract (Gley), crawfish, eel serum, and a host of other extracts, all of which in the dog produce a typical drop in blood pressure and an incoagulable blood.

Pozerski²⁶ found that while the injection of Witte's peptone in treated rabbits had no influence upon the coagulation of the blood, it caused a disappearance of complement and hence of hemolysis in regard to sheep corpuscles. He, therefore, concluded that there was no relationship between the phenomena of incoagulability and loss of complement. This view, however, is not strictly justified inasmuch as change in coagulation time, loss of complement, and production of toxicity are to be considered as expressions of one and the same disturbance involving the highly labile constituents of the blood plasma. The rabbit is not strictly refractory to the anticoagulant action of peptone as he supposed it to be.

Brieger⁴ found that rabbits of about 1 kilo weight when given subcutaneously from 0.5 to 1 gm. of peptotoxin, developed posterior paralysis, became somnolent, and died after some hours. Witte's peptone was tolerated in dose of 20 gm., while a peptone which he prepared by peptic digestion of fibrin was quickly fatal when given subcutaneously in dose of 2 gm.

²⁴ Cinquantenaire de la Soc. de biol., 1899, p. 707.

²⁵ Arch. internat. de physiol., 1906, 3, p. 218.

²⁶ Compt. rend. Soc. de biol., 1913, 74, p. 577.

Fatal results in rabbits following the intravenous injection of Witte's peptone are mentioned by Gley.²³ Hirschfelder noted collapse of the lungs in 2 rabbits killed with peptone, but failed to give any further data. Kumagai,¹⁴ likewise obtained 2 fatal results following the injection of 3.08 and 4.2 gm. per kilo; death was almost immediate, with dyspnea and collapsed lungs. This appears to be the extent of available data regarding the action of peptone on rabbits. It was necessary for the purpose of this study to confirm and extend these observations. It will be shown that Witte's peptone does produce severe and even fatal effects in rabbits, among which drop in blood pressure and decreased coagulability are easily noted. In addition it will be shown that the rabbit may respond with a perfectly typical anaphylactic shock and findings, in which respect it falls in line with the rat and the guinea-pig.²⁷

For the tests given in Table 80, the 10 and 20% solutions of Witte's peptone were prepared in the usual way by dissolving the peptone in salt solution in a water bath at 100 C., and then filtering through paper. In the case of the 30% solution, filtration was found to be very slow and was abandoned in favor of centrifugation at 8000 r.p.m. Even at this speed, the fluid will not be clarified if the temperature is that of the room, the viscosity of the liquid being sufficient to hold up some of the very fine material. This difficulty was overcome by centrifugating the hot solution. The peptone solution employed in all of the injections was perfectly clear. On account of the large bulk of solution to be injected, and especially because of the viscosity, it was found desirable to warm up the solution to 40 C. before use. Obviously, the best result can be expected if the peptone solution is in a condition to mix freely with the blood.

The ordinary syringe was used for the injection of all but Nos. 16 and 17. Since the largest syringe had a capacity of but 20 c.c., it was often necessary to make use of 2 or 3; after the first was discharged, the syringe was separated from the needle, which remained in the jugular, and the next one was attached and the contents injected. In this way, considerable speed was possible, especially if the needle was of fair caliber. In the last 2 tests this method was supplanted by a more convenient procedure in which a 50 c.c. cylinder was equipped with an outflow tube carrying the needle, and an inflow tube attached

²⁷ In a very recent publication (*Jour. Biol. Chem.*, 1917, 29, p. 129), Kuriyama states, that in a few cases 0.5 to 1 gm. of Witte's peptone per kilo of body weight killed the rabbits immediately or in a short time. Prostration, weak heart action, and convulsions were noted.

to a compression bulb. The difference in the injection time of Nos. 16 and 17 was due to the use of different needles, that for the former being short and wide, while that for the latter was very long and fine.

TABLE 80
INJECTION OF PEPTONE INTO RABBITS

Exper.	Rabbit		Peptone		Injection Time (sec.)	Result
	No.	Weight	Intra-venous Injection (c.c.)	gm. per kilo		
A 10% Peptone	1	1,400	14	1	25	Nil
	2	1,220	24.4	2	50	Practically nil
	3	1,235	37.1	3	70	Severe
	4	1,085	32.5	3	70	2/45". Typical shock and autopsy
	5	1,970	59	3	100	Nil
B 20% Peptone	6	1,800	19.5	3	50	Practically nil
	7	1,075	16.1	3	35	Severe
	8	1,525	30.5	4	60	Nil
	9	1,800	45	5	130	Slight
C 30% Peptone	10	1,360	22.6	5	20	7'. Typical shock and autopsy
	11	1,050	17.4	"	12	7' 8". " " " "
	12	1,550	35.7	"	27	3'. " " " "
D 30% Peptone	13	1,350	22.4	5	45	Slight
	14	1,060	17.6	"	15	"
	15	1,510	24.9	"	30	Moderate
	16	2,075	34.5	"	15	3'. Atypical
	17	1,930	32	"	120	Very slight

No. 17 was reinjected 26 minutes later with a like dose and died in 3/20"; the toxicity of the blood is given in Table 95 (Nos. 7 to 9).

The rabbits used for these tests were all new animals. An inspection of the table will show at once that there is a great variation in the resistance of the rabbits to peptone. Of the 17 tests, but 5 proved fatal; and of these, 4 were perfectly typical anaphylactic shocks with characteristic autopsy findings, namely, maximal distention of lungs, heart beating strongly, low blood pressure, the heart free of clot and the blood of normal fluidity. The small intestine in addition to increased peristalsis, showed most intense transverse contractions which fairly wrinkled the entire gut. The lungs were very light pink in color and free from petechiae. The time of death was reckoned from the end of the injection.

The symptoms developed after a slight incubation period of $\frac{3}{4}$ to 1 minute; the respiration became shallow, dyspnea set in, followed by

spasms and convulsions, the animal being thrown; in one instance (No. 11) there was a frothy discharge from the nose. The difficulty in respiration is usually shown by a wheezing or gurgling sound. After the acute effects pass off, the animal becomes more or less somnolent, a condition first observed in dogs by Fano. A very striking symptom, hitherto unobserved, was an exophthalmos which in the fatal cases came on rapidly and was most marked at death; if recovery occurs, this condition disappears in a few minutes. In No. 16, in which, as stated, the injection was made very rapidly, the exophthalmos was extreme; the pupil, at first wide, became constricted, and the iris appeared quite blanched.*

The Lethal Dose.—The lowest amount of peptone which was fatal was 3 gm. per kilo (No. 4). It is rather striking to find that of 9 tests, in which 5 gm. per kilo were given, only 4 proved acutely fatal. This fact clearly shows that the rabbit, like the guinea-pig, is subject to individual variations. This is quite independent of the speed of injection, as will be seen on inspection of the injection times in Exper. D, Table 80, in which the same peptone solution was used and the tests made one after another. It is very likely that the slight effect produced in No. 17 was due to the long injection period. On the other hand, it is also probably true that very rapid injections may cause a speedy death with lungs in collapse, as was the case in No. 16. A moderate speed of about half a minute with thorough intermingling of the peptone and blood is perhaps the condition to be desired.

On comparison of Tables 79 and 80, it will be seen that the rabbit is more resistant to peptone than the rat. Thus, while the least fatal dose for the rat was found to be 2 gm. per kilo, that for the rabbit was 3 gm. per kilo. Compared with the guinea-pig, the least fatal dose for which is 0.3 gm., the rabbit has at least a ten-fold resistance. It is noteworthy that in regard to agar, peptone, anaphylatoxin, and specific anaphylaxis, the rabbit is considerably more resistant than the guinea-pig.

With reference to agar, it should be pointed out that apparently the rabbit is more susceptible than the rat. It is to be noted, however, that only 2 very young rabbits were tested with agar, and it is quite likely that a different result would have been obtained had animals of 1 to 1½ kilos been used, as in the case of the work with peptone.

* It should be mentioned that Camus and Gley (Arch. internat. de pharmacodynamie, 1898, 5, p. 272) observed, at times, a very marked exophthalmos in rabbits injected with cel serum (0.3 c.c.). The other toxic effects of this serum, which have been compared to those of peptone by Delezenne, are without doubt due to the production of anaphylatoxin.

The following tabulation of the least fatal dose in grams per kilo may be of interest:

	Agar	Peptone	Ratio
Guinea-pig	0.009	0.3	1:33
Rabbit	0.016	3.0	1:188
Rat	0.027	2.0	1:74

Immunity.—Like the dog, the rabbit, as well as the guinea-pig, shows an increased resistance to peptone after recovery from the effects of the first injection. If the second injection is given too soon after the first it is likely to cause death, just as in the case of guinea-pigs injected with 0.5 c.c. doses, at intervals of 15 minutes. By way of illustration, it may be stated that Rabbit 17 of Table 80 was reinjected 26 minutes later with the same dose, the injection time being 40 seconds. It died with typical shock in 3 minutes, 20 seconds, but on autopsy the lungs were in collapse, though the heart was beating and the blood was fluid. Transfusion of this blood showed that it was highly toxic (Table 95, B). Again, Rabbit 3 was reinjected 3 hours later, the dose being 4 gm. per kilo, and the injection time, 55 seconds; it was at once limp, showed exophthalmos, and died in 1 minute, 35 seconds after the end of the injection. The autopsy findings were the same as those of No. 17; the blood condition of both will be referred to later.

By contrast, Table 81 presents the results of multiple injections into 2 rabbits of the same series, the second injection being given on the following day. At 20 minutes and at 3½ hours after the third injection of Rabbit 1, blood was drawn from the heart and transferred to a small test tube; the first sign of coagulation was noted at 5 and 3 minutes, respectively, and both samples clotted firmly in 8 minutes. In brief, the blood coagulated in nearly normal time. It was probable that even the interval of 20 minutes was too long and hence after the fourth dosage blood was drawn within 3 minutes after the injection; this remained perfectly fluid for 30 minutes and when reexamined at 40 minutes it showed a slight clot (+) or floater which did not increase during the next 40 minutes. In other words, though the animal showed no symptoms following the fourth injection, its blood was practically incoagulable. The fifth injection was followed by dyspnea, spasms, convulsions, exophthalmos, and death; the autopsy at 2 minutes after death showed collapsed lungs, heart, beating and free of clot, and blood, fluid. The blood transferred to a tube gave no evidence of clotting for 10 minutes, but was solid at 13 minutes.

In Rabbit 6, there was practically no effect observed until after the fifth injection when a transitory exophthalmos and slight respiratory distress developed. After the sixth injection, the eye protrusion came on rapidly, but receded in about 12 minutes; the next injection was followed by spasms and a severe exophthalmos, which, however, again passed off in 10 minutes. This condition recurred after the eighth injection, while the ninth resulted in death. The usual dyspnea, spasms, violent convulsions, and eye protrusion were noted and at autopsy, made within 2 minutes after death, the lungs were collapsed, but the heart was vigorously beating and the blood was fluid. A portion transferred at once to a test tube remained perfectly limpid for 18 minutes; at 19 minutes, the first sign of clot appeared, but even in 30 minutes,

TABLE 81
MULTIPLE INJECTIONS OF PEPTONE INTO RABBITS. IMMUNITY

Rabbit	Number of Injections	Interval	Injection Time (sec.)	gm. per kilo	Result
1 (Table 80)	1	—	25	1	Nil
	2	24 hrs.	20	3	"
	3	22'20"	10	3	"
	4	3 hrs. 50'	40	4	"
	5	22'15"	55	4	1'5". Typical shock
6 (Table 80)	1	—	50	3	Very slight
	2	21 hrs.	25	"	Nil
	3	20'	30	"	"
	4	4 hrs. 25'	35	4	"
	5	8'30"	50	"	Slight
	6	8'55"	55	"	"
	7	17'55"	40	4	Fair
	8	22'20"	40	"	"
	9	6'10"	50	5	2'. Typical shock

coagulation was not complete, the clot being soft and rolling. A second portion of blood, drawn 9 minutes after the first and while the heart still showed a slight beat, gave the first sign of clot in 11 minutes, and a soft sliding clot like that of the first tube, in 22 minutes. By adding to these the interval between the two portions, it will be seen that the two portions behave alike, as has been pointed out in connection with the coagulation of the blood of guinea-pigs.

Coagulation of Blood in Rabbits Injected with Peptone.—Under the preceding heading, several instances have been mentioned going to show that the coagulation changes in rabbit blood may be greatly retarded. It should be added that the blood of Rabbit 3, mentioned

heretofore, was perfectly fluid in the tube for 15 minutes and reexamined at 22 minutes, had a beginning small clot (+), which gradually increased till at 48 minutes it was complete except that it was not firm. Another portion of blood, drawn from the heart 10 minutes after the first lot, began to thicken after 6 minutes, but the process did not go beyond that of a soft clot after which resolution took place. Some of this blood was centrifugated and the clear plasma did not commence to clot for an hour; at the end of 2 hours the clotting was complete (+++++) but soft. This plasma, it may be added, was acutely fatal in dose of 1 c.c.

Rabbit 17 referred to under "Immunity" gave a blood, 4 minutes after death, which showed no sign of coagulation for 35 minutes; at 40 minutes, it developed a very slight clot (+) and this persisted for nearly 2 hours, when it formed a small mass (++) which remained in that condition for 24 hours.

Of especial interest, is the behavior of the blood in those tests in which death occurred after a single injection of peptone. Thus, in No. 4, Table 80, the blood drawn 3 minutes after death showed a small clot (+) at the end of a minute, but this did not increase during the next half hour; in fact it became less so that in an hour and a half it was scarcely to be seen. This resolution or contraction of a very partial clot was repeatedly encountered; it corresponds to the fibrinolysis of Dastre. Another portion of blood, drawn 10 minutes after death, behaved in exactly the same manner. The blood therefore could be said to be practically incoagulable.

In the case of Rabbit 10, Table 80, the blood drawn less than 3 minutes after death remained entirely free of clot for 1 hour; reexamined an hour later it was found to be fully clotted, and showed no retraction for 4 hours.

Rabbit 11 was bled from the heart at 2 minutes after death; the blood gave a small soft clot (+) at 9 minutes, and this did not increase, but after 20 minutes, it began to shrink and the blood appeared to be perfectly fluid and remained so.

In Test 12, the blood was drawn at once after death, and in 11 minutes showed a small clot (+) which gradually increased, so that at 50 minutes it was +++, a soft sliding mass. A second portion drawn 11 minutes after the first gave in 1 minute a small clot (+), corresponding in time to that of the first portion; this clot did not increase, but instead behaved like the others, showing in about 40 minutes a resolution or fibrinolysis.

In Rabbit 16, in which the blood was removed from the heart within 3 minutes after death, no clot was in evidence for 20 minutes; at 22 minutes a very slight clot appeared, which increased a trifle (++) and remained thus, a very soft mass, for more than 2 hours. This result may well be compared with that obtained in No. 13, in which the injection produced but a slight general effect; the heart was exposed and blood drawn 10 minutes after the injection, remained perfectly fluid for 12 minutes, but became solid in 14 minutes (see Table 95, A).

The results of blood examinations of 10 rabbits which received 1 or more injections of peptone have been herein presented. It was deemed important to show, notwithstanding conflicting statements to the contrary, that the rabbit responded to injections of Witte's peptone in essentially the same manner as the dog, the guinea-pig and the rat. There can be no question from the evidence submitted as to this point. The amount of peptone, it is true, must be considerable in order to bring about the disturbance in the rabbit, but when this is done, the result is a typical anaphylactic shock in which the cardinal symptoms, dyspnea, spasms, convulsions, drop in blood pressure, incoagulable blood, and lung distention are to be found.

THE IN-VITRO PRODUCTION OF ANAPHYLATOXIN BY PEPTONE

In order to establish the mode of action of peptone, it was not sufficient to show that when injected it gave rise to symptoms and changes similar to, if not identical with, those produced by anaphylatoxin. Such demonstration could be but the first link in the chain of evidence necessary, and it was imperative to show that peptone like agar could make anaphylatoxin by contact with normal serum, and as a further and final proof, it was essential to demonstrate that such a poison was formed in an animal when injected with peptone.

Previous work had shown that the best reagent for anaphylatoxin-production was rat serum, while that of the guinea-pig, usually employed hitherto, was much less satisfactory, and that of the rabbit still less. Hence, in the solution of the problem which presented itself, rat serum was made the first choice and the results met all expectations. A large number of experiments were likewise made with guinea-pig serum, but the outcome, for reasons which will be duly considered, was not as satisfactory. Inasmuch as the rabbit serum was the least promising, no tests, as yet, have been made with it.

An essential condition for this work is a large pool of fresh serum sufficient for a series of tests, and also for all needed controls. This is necessary because of the fact that the normal serum of a given species is a variable. A further condition which was learned by long experience is the need of safe-guarding against the individual variability of the test animal. This can be done to some extent by injecting the animals in pairs, but even then it is possible that chance has brought together either two susceptible or two resistant animals. It is only from a large series of tests, controlled in every possible way, that trustworthy conclusions can be drawn.

Rat Serum.—Early in the study on anaphylatoxin, some attempts were made to toxify rat serum with peptone so that 1 c.c. of the serum would be fatal. In view of the fact that an unheated unfiltered solution of peptone was more toxic than one that was perfectly clear (Table 70), the first trials were made with such a suspension, and the results are given in Table 82.

The peptone solution was made by adding 10 gm. of Witte's peptone to 100 c.c. of salt solution; the mixture was then heated, with stirring at 60 C. The cloudy, unfiltered solution was used direct. For Exper. A, 3 c.c. of the peptone were added to 6 c.c. of fresh rat serum, and this mixture was tested at once, after incubation at 37 C., as shown in the Table. Expers. B and C were carried out in like manner, the mixtures being 1 + 4 and 1 + 8, respectively. The injection dose in all cases represented 1 c.c. of serum. An inspection of Table 82 will show at a glance that apparently the peptone does render the serum poisonous. It may, however, be objected to Exper. A that the amount of peptone injected (0.5 c.c.) was too near the lethal dose when given by itself, and that, therefore, the results might be due to the direct action of the peptone. This objection, however, does not hold for Expers. B and C. The serum by itself, in the dose employed, is harmless.

The effects observed after the injection of the incubated mixtures of serum and peptone were the same as with sera, which had been treated with trypanosomes or with agar; they were those of anaphylatoxin, that is, dyspnea, spasms, and convulsions. When death was not acute there was a drop in temperature which in No. 9, in 2½ hours, reached 27 C.; at autopsy the usual picture was obtained, namely, maximal lung distention, heart beating and free of clot. The rat serum, toxified with peptone, frequently produces a marked rigidity of the muscles of the abdomen and extremities not seen when the agar anaphylatoxin is used.

TABLE 82
ACTION OF CLOUDY UNHEATED PEPTONE SOLUTION (10%) ON NORMAL RAT SERUM

Exper.	Ratio	Guinea-Pig		Mixture		Result
		No.	Weight	Incubation at 37 C. (min.)	Intra- venous Injection (c.c.)	
A	1:20	1	230	—	1.5	Practically nil
		2	200	7½	"	Severe
		3	195	15	"	2/40". Typical shock
		4	205	30	"	2/55". " "
		5	195	60	"	3/20". " "
		6	190	120	"	0'. " "
B	1:40	7	180	—	1.25	Practically nil
		8	220	7½	"	Severe
		9	185	15	"	6½ hr.
		10	180	30	"	Slight
		11	215	60	"	Practically nil
		12	225	120	"	" "
C	1:80	13	202	7½	1.12	Nil
		14	180	15	"	Very slight
		15	208	30	"	3/45". Typical shock
		16	230	120	"	Nil

Different pools of serum were used for these experiments.

It should be pointed out that the mixtures when tested at once, as soon as made, gave little or no effect, but that the toxicity developed on incubation. In view of the small amount of the serum used as a test dose, it will be seen that even a short incubation of a few minutes gives rise to some poison. Thus, when retested in 7½ minutes, severe shocks were obtained in Experiments A and B, and, in the light of subsequent work, it is likely that had duplicate tests been made, possibly even a fatal result might have developed. The incubation for 15 minutes, in Experiment A did bring about the production of more than an average lethal dose of poison since the 4 consecutive tests made after this time caused typical acute shock and death. On the other hand, in Experiment B, in which the mixture contained but half the peptone of the former, the poison-production was much less since no acute death resulted. The guinea-pig injected with the material after incubation for 15 minutes had an intense, quiet shock; the temperature dropped in 2½ hours to 27 C., and death occurred in 6½ hours. Further, it will be seen that in Experiment C, in which the amount of peptone was still further reduced a typical acute shock and death resulted after incubation for 30 minutes. This outcome, on account of the small amounts, cannot possibly be ascribed either to an initial toxicity of the rat serum,

or to that of the peptone, or to the sum of these two; it leads to the conclusion that the poisonous effect is the result of an interaction of the serum with the peptone, anaphylatoxin being formed.

TABLE 83
ACTION OF CLEAR HEATED PEPTONE SOLUTION (10%) ON NORMAL RAT SERUM

Exper.	Ratio	Guinea-Pig		Mixture		Result
		No.	Weight	Incubation at 37 C. (min.)	Intravenous Injection (c.c.)	
A	1:20	1	225	7½	1.5	Practically nil
		2	205	15	"	Moderate
		3	198	30	"	4'. Intense quiet shock
		4	200	60	"	Moderate
B	1:20	5	190	7½	1.5	Practically nil
		6	205	15	"	Moderate
		7	185	30	"	4'. Intense quiet shock
		8	213	60	"	Slight

The two experiments in Table 83 were made on the same day as Exper. A of the preceding set and were in the nature of controls. The same peptone solution was used, but it was first heated to 110 C. for 15 minutes; this gave a clear liquid with a small flaky deposit, which was shaken up and the resulting mixture used in the first experiment (A). The balance was centrifugated at 8000 r.p.m., and the perfectly clear solution was employed in the second experiment (B). It will be seen that the two series are practically alike. The clear peptone solution can toxify the rat serum, but the toxicity of the mixture does not approach that of the mixture having the peptone suspension. The cloudy suspension is more active *in vivo* and also *in vitro*. Heating, with the resultant dispersion and complete solution of the peptone, clearly reduces its activity. Agar, it is to be noted, is never in perfect solution, since it can be thrown by centrifugation.

The fact that the clear solution of peptone is less active than the cloudy unfiltered suspension corresponds to the observation of Nathan²⁸ that inulin toxifies guinea-pig serum when in a suspended state, but does not do so when warmed to 68 C. for 2 minutes. Evidently, the extreme dispersion of inulin, like that of peptone, is unfavorable to the reaction, conceivably, because the charge or energy of the aggregate is more intense, applied locally, than that of the fully dispersed phase.

²⁸ Ztschr. f. Immunitätsf., 1914, 23, p. 209.

Be that as it may, the fact is that the clear peptone solution (Expers. A and B, Table 83) in the same concentration as that of the cloudy one (Exper. A, Table 82) produces a toxic condition in the serum, less rapidly and to a less degree. The effect after incubation for 15 minutes was but moderate, and, seemingly, incubation for 30 minutes was necessary to produce a fatal dose of poison. Designating as the average, or least fatal dose, that amount of the poison which may kill one-half or less of the test animals, it will be readily understood that the cloudy suspension may produce several multiples of such dose since 4 consecutive tests were fatal, whereas the clear solution can give rise to only a single average lethal dose.

After an interval of 10 months, these tests were repeated in an extended form; the peptone solution was prepared in the same way as for Exper. B of Table 83, except that it was made up with distilled water instead of salt solution. A mixture of 10 c.c. of this peptone and 20 c.c. of rat serum was incubated, and at intervals of 15 minutes, tests were made, the dose being 1.5 c.c. Uniformly mild results were obtained in 9 consecutive tests, made in 2 hours. As the only explanation of this failure seemed to be in the use of distilled water which may not have dissolved as much of the peptone as a salt solution, the experiment was repeated on the following day with a peptone solution prepared in the usual way with salt. The results of this experiment are given in Table 84.

It will be seen from this table that 3 of 12 tests gave acutely fatal shocks. More important was the variability in the results which could not be accounted for by an assumed change in the poison, but rather by a variation in the resistance of the test animals. It indicated that the amount of poison produced was small, a single average lethal dose and not a multiple thereof.

While the foregoing experiments rendered it highly probable that peptone acted on the serum and gave rise to anaphylatoxin, yet the fact that the amount of peptone per dose of serum, as used in most of the tests, was relatively large (0.5 c.c.) made it imperative to meet the objection that the effects observed were due to this amount. Decreasing the quantity of peptone as in Table 82 only partially met the objection. It was desirable to obtain a surely fatal dose, one which would kill all or nearly all of the test animals, in which the amount of peptone would be considered negligible. If a minimal amount of peptone did not produce a surely fatal dose of poison in 1 c.c. of serum, it possibly

would do so were the amount of serum doubled or trebled. Ordinarily, normal rat serum can be injected in dose of about 5 to 6 c.c. without any serious effect, but, on the other hand, it is possible to prepare such serum so that in dose of 3 to 4 c.c. it will be acutely fatal (p. 533). Consequently, to be perfectly safe, it was decided not to increase the amount of serum per dose beyond 2 c.c.

TABLE 84

ACTION OF CLEAR HEATED PEPTONE SOLUTION (10%) ON NORMAL RAT SERUM. VARIATION IN RESISTANCE OF GUINEA-PIGS

Ratio	Guinea-Pig		Mixture		Result
	No.	Weight	Incuba- tion at 37 C. (hrs.)	Intra- venous Injection (c.c.)	
1:20	1	195	$\frac{1}{4}$	1.5	Slight
"	2	183	$\frac{1}{2}$	"	3'15"
"	3	192	$\frac{3}{4}$	"	Moderate
"	4	186	1	"	5'20"
"	5	209	$1\frac{1}{4}$	"	Practically nil
"	6	196	$1\frac{1}{2}$	"	Good shock
"	7	207	$1\frac{3}{4}$	"	Practically nil
"	8	189	2	"	" "
"	9	181	$2\frac{1}{4}$	"	Fair shock
"	10	177	$2\frac{1}{2}$	"	Very slight
"	11	208	$2\frac{3}{4}$	"	Slight
"	12	176	3	"	3'45"

The experiments which were made under these conditions did not at once yield the desired result and some idea of the effort expended in this final series of tests may be gathered from the fact that about 125 guinea-pigs were used. Eventually, the chief difficulty was found to lie in the serum. In the production of anaphylatoxin out of rat serum by means of agar, it mattered little whether the serum was used perfectly fresh or after being iced for 24 hours; in the peptone work, however, it did make a great deal of difference in results, according as the serum was fresh or had been iced for from 6 to 9 or 12 hours. Seemingly, the iced serum tends to form a stable condition which the powerful action of agar can disturb, but which the weaker peptone is unable to do. A similar condition is seen when normal rat serum, which has been heated at 50 C. for 15 minutes, is subjected to the action of agar, or of distilled water (Part IX).

By way of illustrating the statement just made, the following results of a series of experiments made with one and the same pool of serum are presented. The blood from 20 rats bled in the morning and early afternoon, each being separately defibrinated, was pooled and centrifuged.

gated at 8000 r.p.m. and the 76 c.c. of serum thus obtained were pooled in a small beaker and placed in cracked ice. The experiments were performed in the evening, the first being made 6½ hours after the last bleeding. For this first experiment, a mixture of 2 c.c. of 10% peptone and 16 c.c. of the serum was made and iced for 10 minutes, after which it was placed at 40 C. The test dose of the mixture was 2.25 c.c., and the injection time 4 to 7 seconds. The tests were made in pairs, one just before, and the other after incubation for 5, 15, and 30 minutes, making 4 pairs or 8 tests. After incubation for 5 minutes, one test gave a severe shock while its companion was less affected; at 15 minutes, both tests resulted in acute death; at 30 minutes, one test was fatal while the other showed but slight effects.

The results of this first experiment were very good, since the serum had become toxified within 15 minutes. By way of control to show that the observed toxicity was not due to the serum, a like number of guinea-pigs (8) were injected at once with the same pooled serum, each receiving 2 c.c.; the effects were slight or nil, as was expected. Accordingly, as a check, the first experiment was repeated exactly, with the same serum, peptone, and time conditions. Of the 4 pairs of tests or 8 guinea-pigs, only 2 gave severe shocks and there were no deaths. This second experiment, which proved a complete failure, was begun 1½ hours after the first, and as the result was so unexpected, it was repeated a third time under identical conditions, the only variation being in the age of the iced serum. The test was begun 2½ hours after the first. Of 8 guinea-pigs, tested as 4 pairs, 5 had but slight effects, 2 received a severe shock, and 1 had an atypical shock and died in 5 minutes, 30 seconds.

As a further control test, 3¼ hours after the first experiment, a mixture of 1 c.c. peptone and 12 c.c. of the same pooled serum was made; 2 tests were made at once, and 2 after incubation for 15 minutes. The dose injected was 3.25 c.c., representing 3 c.c. of serum. The effect in the first pair was practically nil, while that of the second was moderate. This control and the 2 check experiments clearly showed that the serum was not as reactive as it was at first. Apparently, prolonged exposure to air and to 0 C. was responsible for the change.

In order to test this conclusion, on the following day, a pooled rat serum was prepared, and after being kept in cracked ice for 5 hours, a portion of it was treated with peptone and incubated under the same

conditions as prevailed in the experiments of the day before. After incubation for 5 minutes, the paired test gave 2 severe shocks; at 15 minutes there was 1 acute death and 1 near-kill, and at 30 minutes both guinea-pigs died of acute shock. This experiment was very satisfactory as a demonstration of poison-production, since the mixture tested on a pair of guinea-pigs before it was incubated had very little effect. As a check, the experiment was repeated under the same conditions as before, except that the serum had been iced now for 6 hours. The peptone-serum mixture after being iced for 10 minutes was unexpectedly active, since the usual dose of 2.25 c.c. caused 1 acute death and 1 very severe shock. On incubation for 5 minutes, the mixture gave 2 severe shocks; at the 15 minute test, there was 1 death and 1 moderate shock; at the 30 minute test, there was again 1 death and 1 fair shock. Evidently, in this second test, the full amount of poison was produced very early, since incubation showed no increase. Seemingly, the serum which had been iced for from 5 to 6 hours was fairly reactive to peptone, the result being very much like that of the first experiment of the preceding day.

For the next experiment, it was decided to use as fresh a serum as possible. For this purpose 17 rats were bled in half an hour; each blood was separately defibrinated with a rod, and the pooled mass was then centrifugated at 8000 r.p.m. The pooled serum was at once iced and was used for the first experiment within $2\frac{3}{4}$ hours after the start of bleeding. The results of this experiment are noteworthy, especially since the rats were controlled in every possible way (Table 85).

Whatever the cause, the serum in this experiment proved to be very reactive, since the mixture of peptone and serum became toxified during the icing period of 10 minutes. On subsequent incubation, apparently the full amount of poison was soon produced, since all the test animals died of typical shock. This result was so striking that it was at once controlled with 4 series of checks, in which the same pooled serum or peptone were used. Control 1 was started within 45 minutes after the injection of No. 1; Control 2 within 1 hour 45 minutes; Control 3 within 1 hour; and Control 4 within 1 hour 25 minutes, the entire experiment being put through inside of 2 hours from the first injection.

Control 1 is noteworthy, since it shows that in Nos. 7 and 8 a lethal dose of anaphylatoxin was produced within a very short time, 45 seconds. This speed of production was such that it raised the question as

to whether the poison was made in the mixture before injection, or was developed in vivo after such injection. In order to determine this point, Control 2 was made.

In Control 2, the guinea-pig was given an injection of 2 c.c. of the serum, the syringe was then detached from the needle, which remained

TABLE 85
ACTION OF CLEAR HEATED PEPTONE SOLUTION (10%) ON NORMAL FRESH, RAT SERUM

Exper.	Ratio	Guinea-Pig		Mixture			Result
		No.	Weight	Incubation		Intra-venous Injection (c.c.)	
				At 0 C. (min.)	At 40 C. (min.)		
A	1:80	1	200	10	—	2.25	Good shock
	"	1a	200	"	—	"	4'
	"	2	200	"	5	"	3'
	"	2a	200	"	"	"	3'56"
	"	3	195	"	15	"	3'
	"	3a	195	"	"	"	22'
	"	4	200	"	30	"	3'34"
	"	4a	197	"	"	"	4'20"
Control 1	1:80	5	185	—	—	2.25	Slight
	"	6	200	—	—	"	Moderate
	"	7	180	—	—	"	4'29"
	"	8	198	—	—	"	3'39"
Control 2	1:80	9	194	—	—	2 + 0.25	Practically nil
	"	10	195	—	—	" "	41'
	"	11	204	—	—	" "	Slight
	"	12	192	—	—	" "	Slight
Control 3		2 c.c. of serum were injected into each of 5 guinea-pigs; result nil or slight					
Control 4		2.25 c.c. of peptone-salt solution (1:8) injected into each of 5 guinea-pigs; result nil					

* For each test the mixture was made separately (2 c.c. serum + 0.25 c.c. peptone). The time of reaction, that is, from start of mixing to end of injection, was but 41 to 45 seconds.

† In this control set, the serum and peptone were injected consecutively, the injection time for the 2 c.c. of serum was 4 to 10 seconds, while that for the 0.25 c.c. of peptone was but 2 seconds; the total time from the start of the injection of the serum until the end of that of the peptone was 13 seconds for Nos. 10 and 12; 14 seconds for No. 11; and 18 seconds for No. 9.

in the vein, and another with the peptone solution was connected. The speed of the entire operation is indicated in the footnote to the table. It will be seen from this control set that the very rapid and consecutive injection of the two fluids should give a summation of toxicity of the two fluids and in addition should express that which might result from the in-vivo reaction of the two components. While some

in-vivo production is indicated (No. 10), the results conclusively show that the acute deaths in Exper. A, and also those of Control 1 (Nos. 7 and 8) are due to the in-vitro interaction of the peptone and serum. Controls 3 and 4 were made to show the innocuousness of the serum and of the peptone when given separately.

It does not seem possible to interpret the results of Exper. A, in view of the controls made, in any other way than that the peptone itself is not toxic, but that when brought into contact with a reactive serum it gives rise to a poison, namely, anaphylatoxin.

It has been shown above that the reactivity of the serum depends in part upon the age of the serum, and perhaps on the length of exposure to air. An additional factor, however, is involved, for, in the next experiment, which was made with a serum, 35 minutes after the start of bleeding (as fresh a serum as possible), a test of 2.25 c.c. at the end of an incubation of 5 minutes was nil, while of a pair of tests made at 15 minutes, one gave an acute death and one but a slight effect. In another experiment with a serum, within 2½ hours after start of bleeding, the result was somewhat better, for, after icing the mixture (1:8) for 10 minutes, one test gave a severe shock, while that of its companion was also marked; after incubation for 5 minutes, one of a pair of tests gave acute death, the other but a slight effect; after 15 minutes a similar result was obtained, one death and one slight shock.

The excellent results of the experiment, recorded in Table 85, contrasted with those just mentioned, showed that the age of the serum was not the sole factor. Some other condition contributed to the reactivity of the serum; this might be inherent in blood of the individual animal, or it might lie in the mode of defibrination.

Peptone is at best a weak toxifying agent, and the poisonous effects noted in Table 85 and elsewhere must be looked upon as the sum of the primary or initial toxicity of the serum, and of the anaphylatoxin produced by the action of the peptone. Were the primary toxicity zero (which it never is, since the untreated serum is often fatal in dose of 6 c.c.), then the peptone would have to induce the production of a full dose of poison; on the other hand, if the primary toxicity amounts to two-thirds of a fatal dose, then the peptone would have to produce only the remaining fraction, thereby raising the toxicity over the threshold, as it were.

It will be shown in Part VIII that contact of a serum with its clot tends to increase its toxicity; this fact was made use of in the next

and final experiment. The blood of 6 rats was transferred to a common cylinder (50 c.c.) and defibrinated with a rod, after which the blood with the clot was kept at room temperature for 1 hour; it was then centrifugated, yielding 18 c.c. of pooled serum, which was then iced. A mixture, consisting of 1.5 c.c. peptone and 12 c.c. of the serum, was made, and after icing for 10 minutes, a pair of guinea-pigs were injected with the usual dose of 2.25 c.c., with only a slight or moderate effect. The first injection was made within 2½ hours after the start of bleeding. The mixture then incubated and tested after 5 minutes gave 1 acute death and 1 good shock; retested after 15 minutes, it gave 2 acute deaths. Three control tests, similar to Nos. 5 to 8 of Table 85, showed at most only a slight effect. The results of this experiment were excellent, almost as good as those given in Table 85. A fresh serum and a certain degree of primary toxicity appear to be conditions which assist in bringing out the action of peptone.

Guinea-Pig Serum.—The statement has been repeatedly made by Friedberger²⁹ that he and Mita obtained anaphylatoxin with Witte's peptone. The reference given by him does not appear to contain any information on that point other than the mere assertion that "Witte's peptone by the addition of complement becomes more poisonous." Besredka, Ströbel, and Jupille³⁰ in their work on "peptotoxine" gave some experiments in which 0.5 c.c. of 10, 2, and 0.2% peptone solutions were incubated, each with 3.5 c.c. of guinea-pig serum. The mixtures, after being kept for an hour at 37 C., and for 18 hours at room temperature, were found to be nontoxic, and from this they concluded that peptotoxin was not formed by the action of serum on the liquid peptone, and that the latter had to be in a particular physical state in order to be acted upon by the serum. According to their view, which corresponds with that of Friedberger, peptone is changed by the serum into peptotoxin, and this they practically identified with the bacterial anaphylatoxin. Without doubt, the peptotoxin of Besredka is anaphylatoxin produced by agar action.

It follows from this that the production of peptone anaphylatoxin in guinea-pig serum has not been demonstrated, though Friedberger claims to the contrary. Furthermore, it will be shown that this result is not as easily attained as in the case of rat serum, and the reason for this comparative failure must be made clear.

²⁹ Deutsch. med. Wehnschr., 1911, 37, p. 429. Ztschr. f. Immunitätsf., 1911, 10, p. 381; 1913, 17, p. 507; 1914, 22, p. 522.

³⁰ Ann. de l'Inst. Pasteur, 1913, 27, p. 192.

It has been demonstrated that relatively large amounts of peptone must be injected into guinea-pigs in order to produce fatal results. The clear peptone solution in dose of 0.6 c.c. may kill one-half of the test animals (Table 75), and the same result is obtained with 1 c.c. (Tables 71 and 72); even 1.5 c.c. may occasionally fail to produce a fatal shock. On the assumption that anaphylatoxin is produced when peptone is injected into the animal, it follows that 0.6 to 1 c.c. produces only an average lethal dose of the poison, since but one-half of the animals die. It may be assumed that the test guinea-pigs weighing 200 gm. contain 20 gm. of blood or 10 c.c. of plasma which furnishes the matrix for an average lethal dose of the poison. If serum is as reactive as plasma, it should be possible to make this amount of poison in vitro by bringing together 0.6 c.c. of peptone and 10 c.c. of the serum; but the injection of this amount of serum is out of question, since 6 c.c. and even less of normal guinea-pig serum at times may be fatally toxic.

The addition of 0.6 c.c. of 10% peptone to 10 c.c. of plasma represents a ratio of 1:166 (dry peptone), and since this gives rise to but an average lethal dose of poison in corpore, it follows that a mixture of this amount of peptone and serum might be expected to produce the same dose or less, and this being the case, the injection of half of such mixture (5.3 c.c.) would have little or no effect. In order to obtain results with in-vitro mixtures, it is therefore necessary to increase the amount of peptone which is added to the serum (that is, the ratio of dry peptone to serum) and to use as large a dose of such mixture as possible.

The amount of peptone which can be added to a serum is limited, however, by the fact that it is fatally toxic in a certain dose. Thus, 0.3 to 0.5 c.c. of a 20% solution when given by itself will kill one-half of the test animals, and in view of this it would be necessary to use the peptone in less amount were it not for the fact, already pointed out in Tables 75 and 76, that serum has a distinctly neutralizing action as regards the peptone. A mixture of serum and peptone may therefore actually be less toxic than the peptone solution when injected by itself. Just how far it is safe to increase the amount of peptone in such a mixture cannot be stated, but the limit is probably near 0.75 c.c. of a 20% solution, which, if injected alone, would be an almost surely fatal dose. It follows from this that the amount of peptone present in a given dose of mixture must be at or below this figure, and furthermore,

rigid controls must show that such mixture is initially nontoxic (see controls in Table 85).

The dose of serum to be employed in these tests should be as large as possible (3 to 5 c.c.), for it has been shown hitherto that guinea-pig serum is less easily toxified by agar than is rat serum. It is quite futile to attempt to toxify this serum with peptone so that it will be fatal in dose of 1 c.c. — an experience gained quite early in this investigation. On the other hand, when large doses are to be injected, it must be remembered that the serum possesses a primary toxicity, which is a variable depending in part on the animal, and in part on the mode of defibrination and treatment. M. Wassermann and Keysser³¹ were perhaps the first to note that a pooled guinea-pig serum could be toxic in dose of 4 or 3 or even 2 c.c., but their statement, being contrary to usual experience, was not given credence. It will be shown in Part VIII that normal guinea-pig serum can be obtained which will be fatal in dose of 3 or even 2 c.c. That rat serum may be toxic in dose of 3 c.c. was shown in Part I.

One or two tests are not sufficient to control the toxicity, especially when it concerns a dose of 5 or 6 c.c., for it must be borne in mind that guinea-pigs vary greatly as to their susceptibility to anaphylatoxic serum (Table 45), to peptone (Table 72), and to normal sera (Part VIII). If possible, fully as many animals should be used for the control tests as for the experiment proper. The trio of pitfalls to guard against in this work is the toxicity of the peptone, that of the serum, and the susceptibility of the guinea-pig.

A pooled serum, to a certain extent, neutralizes the differences which are inherent in the individual sera, but it does not follow that even pooled sera will behave alike. It has been pointed out on page 695 that different pools of rat serum show an unlike behavior to peptone; the reactivity depending upon the freshness, and upon the degree of primary toxicity since the visible reaction represents the sum of the auto-anaphylatoxin, formed in the blood especially during coagulation, and of the poison produced by the peptone. Hence, the apparent production of a fatal dose of poison by peptone is easier when the amount of serum is fairly large and the inherent toxicity is relatively high; under these conditions an increase in toxicity is more readily detected than would be possible if the initial toxicity were strictly nil.

³¹ *Folia serologica*, 1911, 7, p. 243. *Ztschr. f. Hyg. u. Infektionskr.*, 1911, 68, pp. 541, 544. *Centralbl. f. Bakteriol.*, 1911, 50, Beiheft, pp. 52, 72, 78.

Though the mode of action of peptone as regards rat serum has been established by the results just given, it is equally important to show that guinea-pig serum behaves in the same way. If it was difficult to arrive at conclusive results with rat serum, it was even more so, with that of the guinea-pig. Because of this fact it was deemed best to present at some length the consideration of the conditions underlying the problem, and for the same reason it is desirable to give all of the experimental data.

In Table 75 will be found the results of an attempt at toxifying a serum, using 0.3 c.c. of peptone solution per 3 c.c. of serum, the ratio for dry peptone being 1: 50. On comparison with Table 76, where the peptone ratio was 1: 30, it will be seen that, contrary to expectation, the latter concentration appears to be less active than the former; but since a different pool was used for each set, it is more likely that the cause of the variation lies in the sera.

In the belief that an increase in the amount of serum would be advantageous, the peptone remaining the same, Expers. A, B, and C of Table 86 were then made. The same pooled serum was used for Expers. A and B. It will be seen that there is no particular difference in the results of the two experiments, though the mixture in Exper. A was iced and then incubated, while that in Exper. B was placed directly at 40 C. Expers. C and D were made with another pooled serum, 2 days later, and concerning the former, it is to be noted that the outcome was practically nil. Hence the results of these 3 experiments were not as good as those given in Table 75. This may be ascribed either to a difference in the sera or to the fact that the ratio of dry peptone to serum (1:78) was less than before. Exper. D is noteworthy when contrasted with Exper. C, since in this case the same serum was rapidly toxified by a slight increase in the amount of peptone (1:45); the concentration was, therefore, approximately that used in Table 75, but the dose was increased to 5 c.c. It would appear from this that good results can be expected when large doses of serum are used.

When used in increased dose, the peptone-serum mixture 1:78 may, however, give very fair results, as seem to be indicated in Exper. B. Table 87, where the conditions were similar to those in A and C of the preceding table. The first 2 tests of Exper. B served as preliminary controls and, as such, developed only a slight shock; it might be assumed from this that the subsequent 4 fatal results indicate the for-

mation of anaphylatoxin. While probably this did occur and the poison formed was but an average lethal dose, still in the absence of a large number of controls (9), the conclusion is not quite justified. Meticulous care must be taken to exclude the factor of individual susceptibility in the test animals. Attention should be called to Exper. A, where the same serum was used, but in a greater dose, the ratio being 1:95; seemingly there was a rapid increase in toxicity in 1a as compared with No. 1. This same concentration and dose was used in the experiment given in Table 88, and the result there obtained when contrasted with that just mentioned shows clearly that the variation is due to a difference in the sera.

TABLE 86

ATTEMPT AT ANAPHYLATOXIN-PRODUCTION. THE DOSE REPRESENTS 4.7 C.C. GUINEA-PIG SERUM AND 0.3 C.C. 20% PEPTONE, EXCEPT IN EXPER. D., IN WHICH IT IS 4.5 AND 0.5 RESPECTIVELY

Exper.	Ratio	Guinea-Pig		Mixture			Result
		No.	Weight	Incubation		Intra-venous Injection (e.e.)	
				At 0 C. (min.)	At 40 C. (min.)		
A	1:78	1	192	18	—	5	Practically nil Slight Moderate Severe 6'52"
	"	1a	"	"	—	"	
	"	2	203	"	7	"	
	"	3	"	"	15	"	
	"	4	205	"	30	"	
B	1:78	1	205	—	—	5	Slight Moderate " 7'5" Very slight
	"	2	194	—	15	"	
	"	3	197	—	30	"	
	"	4	"	—	45	"	
	"	5	192	—	60	"	
C	1:78	1	190	17	—	5	Nil " Practically nil Very slight " "
	"	1a	"	"	—	"	
	"	2	200	"	7	"	
	"	3	195	"	15	"	
	"	4	193	"	30	"	
D	1:45	1	195	16	—	5	3' 3'55"
	"	1a	197	"	—	"	

The same pooled serum was used for A and B; it was from 5 guinea-pigs bled about 5 hours before. Another pool was employed in C and D, and was from 8 guinea-pigs bled also about 5 hours before. Injection time, 5 to 9 seconds.

A further attempt at toxifying with the same amount of peptone (0.3 c.c.), but increasing the dose of serum giving the ratio 1:95, is given in Table 88. The conditions were essentially the same as in Exper. A of the preceding table, and as pointed out, the difference in

results can only be due to a variation in the quality of the two pooled sera employed in these experiments. Although the amount of serum in this case was 5.7 c.c., it was lacking in something, since the peptone (0.3 c.c.) was able to toxify but 1 of 8 tests.

TABLE 87

ATTEMPT AT ANAPHYLATOXIN-PRODUCTION. THE DOSE REPRESENTS 0.3 C.C. 20% PEPTONE AND 5.7 OR 4.7 C.C. GUINEA-PIG SERUM

Exper.	Ratio	Guinea-Pig		Mixture			Result
		No.	Weight	Incubation		Intra-venous Injection (c.c.)	
				At 0 C. (min.)	At 40 C. (min.)		
A	1:95	1	200	1	—	6	1 hr.
	"	1a	204	2	—	"	3'57"
B	1:78	1	193	1	—	5	Slight
	"	1a	194	2	—	"	"
	"	2	201	30	—	"	Moderate
	"	2a	200	"	—	"	4'20"
	"	3	205	"	7	"	Slight
	"	3a	200	"	"	"	4'16"
	"	4	200	"	15	"	Moderate
	"	4a	194	"	"	"	3'10"
	"	5	200	"	30	"	3'40"
	"	6	198	"	45	"	Good
	"	7	"	"	60	"	Moderate

A single pooled serum from 6 guinea-pigs was used for this set. Exper. A was made within 3 hours after bleeding, and B 3 hours after A. Injection time, 5 to 8 seconds.

TABLE 88

ATTEMPT AT ANAPHYLATOXIN-PRODUCTION. THE DOSE REPRESENTS 0.3 C.C. 20% PEPTONE AND 5.7 C.C. GUINEA-PIG SERUM

Exper.	Ratio	Guinea-Pig		Mixture			Result
		No.	Weight	Incubation		Intra-venous Injection (c.c.)	
				At 0 C. (min.)	At 40 C. (min.)		
A	1:95	1	200	2	—	6	Very slight
	"	1a	205	"	—	"	"
	"	2	205	28	—	"	3'37"
	"	3	206	"	7	"	Very slight
	"	3a	201	"	"	"	"
	"	4	192	"	15	"	Slight
	"	5	203	"	40	"	"
	"	6	206	"	60	"	Very slight

In this experiment, the pooled serum from 4 guinea-pigs was used about 4 hours after start of bleeding. Injection time, 8 to 12 seconds.

In the experiment given in Table 76, where the dose consisted of 0.5 c.c. peptone and 3 c.c. serum, ratio 1:30, the results were rather indifferent; this also could be ascribed to a feebly reactive serum and

the small amount used. Essentially the same results are met with in Experiments C and D of Table 90, where the same dosage was employed. On the other hand, in Experiment D of Table 86, where the dose of peptone was the same but the amount of serum increased, ratio 1:45, the results were striking, and because of this the experiment given in Table 89 was planned. In the belief that less peptone and more serum would be advantageous, the ratio of 1:100 was employed, but the result was not marked and might well be explained by the individual susceptibility of the animals.

TABLE 89

ATTEMPT AT ANAPHYLATOXIN-PRODUCTION. THE DOSE REPRESENTS 0.5 C.C. OF 10% PEPTONE AND 5 C.C. OF GUINEA-PIG SERUM

Exper.	Ratio	Guinea-Pig		Mixture			Result
		No.	Weight	Incubation		Intra-venous Injection (c.c.)	
				At 0 C. (min.)	At 40 C. (min.)		
A	1:100	1	190	10	—	5.5	Moderate
	“	1a	194	“	—	“	Very slight
	“	2	190	“	5	“	3/18"
	“	2a	“	“	“	“	Fair
	“	3	195	“	15	“	Slight
	“	3a	“	“	“	“	Very slight
	“	4	193	“	30	“	4/8"
	“	4a	198	“	“	“	Severe
Control* 1		5	200	—	—	5.5	Very slight
		6	202	—	—	“	Severe

* For each control the mixture was made separately and injected at once; the time of reaction, that is, from start of mixing to end of injection, was 48 and 52 seconds respectively. These tests were made preliminary to the experiment proper and not at the close.

A pooled serum from 5 guinea-pigs was used 3 hours after start of bleeding. Injection time, 8 to 12 seconds.

For Experiments A and B of Table 90, the amount of peptone was increased to 0.75 c.c., a rather dangerous procedure, since this amount, given by itself, is usually fatal, as will be seen on reference to Controls 2 and 3 of Table 91. The serum, however, has a distinct neutralizing action as is evident from Experiment B, where the mixture was incubated directly without previous icing. The subacute deaths and one failure to kill show that an antagonism exists. This condition is also to be seen in Control 1 of Table 91; unfortunately, no control of this kind was made in the experiment of Table 90 on account of the pool of serum being exhausted. Expecting that the mixture of 0.75 c.c. peptone and 3 c.c. of serum would have little effect or at most cause subacute deaths, when injected as soon as made, it seemed that on further icing or incubation the peptone would induce the production of enough

anaphylatoxin to make it acutely fatal. This result was apparently obtained in Exper. A of Table 90, for it will be observed that while the first test resulted in a delayed death, the other three tests produced acutely fatal shocks. This result is in marked contrast with that of Exper. B, where incubation at 40 C. resulted in a distinctly weaker action, indicating that the temperature of 40 was unfavorable for the poison-production. Expers. C and D of the same table were made with the same serum and peptone, hence the results are comparable with those of A and B; it appears that 0.5 c.c. of peptone is not as active as 0.75 c.c., the amount of serum being the same.

TABLE 90

ATTEMPT AT ANAPHYLATOXIN-PRODUCTION. THE DOSE REPRESENTS 3 C.C. OF GUINEA-PIG SERUM AND 0.75 AND 0.5 C.C. OF 20% PEPTONE, RESPECTIVELY

Exper.	Ratio	Guinea-Pig		Mixture			Result
		No.	Weight	Incubation		Intra-venous Injection (c.c.)	
				At 0 C. (min.)	At 40 C. (min.)		
A	1:20	1	196	15	—	3.75	62'
		2	197	19	—	"	6'55"
		3	195	21	—	"	4'
		4	195	24	—	"	3'50"
B	1:20	5	195	—	15	"	14'7"
		5a	193	—	"	"	Slight
		6	197	—	30	"	18'51"
		6a	200	—	"	"	1 hr. 45'
C	1:30	7	203	16	—	3.5	Fair
		7a	203	"	—	"	Good
D	1:30	8	190	—	12	"	Severe
		8a	190	—	"	"	"
		9	190	—	30	"	Moderate
		9a	193	—	"	"	Very slight

A single pooled serum from 5 guinea-pigs was used for these experiments. The tests were made 4 to 5 hours after starting to bleed. The injection time was 3 to 8 seconds.

In the hope that a more decisive result could be obtained by the employment of mixtures consisting of 0.75 c.c. of 20% peptone and 4.5 or 5.5 c.c. of guinea-pig serum, a more extensive experiment was planned. For this purpose, the pooled serum from 11 guinea-pigs (101 c.c.) was used, and the first tests were made 4 hours after starting to bleed. Of 5 preliminary controls, similar to Nos. 5 to 8 of Table 91, each receiving the dose of 5.25 c.c. as soon as mixed, 1 died of acute shock and 1 of subacute shock. Accordingly, a mixture of 6 c.c. of peptone and 36 c.c. of the pooled serum was made (ratio 1:30); this was iced and tested in pairs at 15, 30, 45, and 60 minutes, but of the

8 tests only 2 resulted in acute death. In the belief that a better result might be obtained by increasing the amount of serum, another mixture of 3 c.c. peptone and 22 c.c. of the same pooled serum was made (ratio, 1:37) and iced; of a pair of tests made with 6.25 c.c. of the mixture, after icing for 30 minutes, 1 gave an acute death; of another pair of tests made after icing for 35 minutes and incubating at 40 for 15 minutes, none developed more than a slight effect.

The disappointing results of this series clearly indicated that the serum was not as reactive as in Experiments D of Table 86, or A of 87, or A of 90. A better outcome was looked for in the next trial, where a perfectly fresh pooled serum was used within $1\frac{3}{4}$ hours after starting to bleed. The results are given in Table 91. The mixture employed in Experiment A consisted of 3 c.c. of peptone and 24 c.c. of the pooled serum, ratio 1:40, and the dose was 6.75 c.c. Although 6 c.c. of serum, given by itself, may cause acute death in a fraction of the test animals, and similarly 0.75 c.c. is an almost surely fatal dose (Nos. 9 to 14), yet the two mixed and injected at once (Control 1) caused but 1 acute death, the others being delayed (see Table 76). It may be added that these controls were made while the mixture was icing for Test 1. The results obtained in the control tests 5 to 8 were just what was desired, since it was hoped that a further slight increase in toxicity, due to an inducing action of peptone, would push the effect over the threshold, as it were, and yield a number of acute kills. This expectation was realized in Tests 2 and 3; whether the delayed death in No. 4 was due to the incubation at 40 C. or to a more resistant animal cannot be stated.

In all of these tests when incubation was resorted to, it was done at 40 C. in the belief that a higher temperature would be more favorable. The results, however, point to a distinctly destructive action upon the poison, probably due to the action of the peptone. It will be seen from Tables 86, 87, 90, and 91, that the mixtures appear to become toxic, even without incubation, and given a reactive serum, better results may be expected by keeping mixtures at 30 instead of at 40 C.

Summation.—Since peptone, when injected intravenously, produces acute shock and rapid death, it must be concluded that the anaphylatoxin production is rapid within the animal, and if so, it should be nearly as rapid *in vitro*. Peptone, however, is not very poisonous, or better stated, it does not readily produce poison; this is seen in the fact that relatively large doses must be injected into animals in order to produce a result. It has been shown on page 683 that its action on

animals, compared with that of agar, is weak. It was shown that in the case of the guinea-pig, 33 times as much of peptone as of agar must be given; similarly, the rat required 74 times as much. This great difference in the action of the two substances is reproduced in the test tube, and it must be correlated with the physical state of each. While the agar is in suspension, the peptone is in true solution, and in this state it does not possess a very marked action. The fact that a peptone suspension is more toxic than a clear solution was pointed out in connection with Table 70. It was also pointed out that Nathan made a similar observation concerning inulin which in suspensoid state toxified serum, but when dissolved by heating at 68 C. for 2 minutes, it was without effect.

TABLE 91

ATTEMPT AT ANAPHYLATOXIN-PRODUCTION. THE DOSE REPRESENTS 0.75 C.C. OF 20% PEPTONE AND 6 C.C. OF GUINEA-PIG SERUM

Exper.	Ratio	Guinea-Pig		Mixture			Result
		No.	Weight	Incubation		Intra-venous Injection (c.c.)	
				At 0 C. (min.)	At 40 C. (min.)		
A	1:40	1	187	30	—	6.75	16'40"
		2	190	45	—	"	3'45"
		3	190	50	7	"	2'50"
		4	188	"	19	"	28'50"
Control 1	1:40	5	187	—	—	"	23'
		6	195	—	—	"	12'24"
		7	200	—	—	"	14'44"
		8	187	—	—	"	5' 6"
Control 2		9	189	—	—	"	3' 9"
		10	187	—	—	"	2'32"
		11	190	—	—	"	3'40"
Control 3		12	185	—	—	0.75	3' 9"
		13	187	—	—	"	3'32"
		14	184	—	—	"	7'17"

A single pooled serum from 5 guinea-pigs was used for these tests, No. 1 of which was made 1½ hours after start of bleeding. The injection time for the large dose was 10 to 13 seconds, and about 8 seconds for the other.

For each test of Control 1, the mixture was made separately (6 c.c. serum + 0.75 c.c. peptone); the time of reaction, that is, from start of mixing to end of injection was from 51 to 68 seconds, that of No. 7 was 1 min. 26 seconds.

For each test of Control 2, a separate mixture of 6 c.c. of salt solution and 0.75 c.c. peptone was made. For Control 3, the undiluted peptone was used.

In order that the clear solution of peptone shall produce the disturbance in vivo which leads to poison-production, it must be used in considerable amount, and the same condition holds true for its in-vitro action. The rat serum which is extremely reactive with agar can also be toxified with peptone, but relatively the amounts of serum and peptone must be greatly increased. Thus, the excellent results in Table 85

were obtained with a peptone-serum mixture in which the ratio of dry peptone to rat serum was 1:80, but the amount of poison produced was not large, since 2 c.c. of the serum had to be injected in order to kill. When it is recalled that an agar-serum mixture (No. 3) in which the ratio is 1:40000 (Table 49) can toxify rat serum in 15 minutes so that 0.25 c.c. is fatal, it will be realized that peptone is decidedly inferior to agar as a toxifying agent. And, further, in view of this fact, it should be clear that very marked results with guinea-pig serum can hardly be expected.

A comparison of the agar-serum ratio 1:40000 with that of the peptone-serum mixture (1:80) used in the afore-mentioned experiment will make it evident that agar is 500 times as active as peptone as to in-vitro mixtures. Again, it has been pointed out in Part II that guinea-pig serum can probably be toxified in dose of 3 c.c. by a sol-gel mixture, No. 3, in which the ratio of agar to serum is 1:40000. Certainly, Mixture 4 (Table 23), having a ratio of 1:8000, readily toxifies it in 1.5-c.c. dose (Tables 24, 25). On comparing these ratios with that employed in Table 91 (1:40), it will be seen that, as to guinea-pig serum, agar is from 200 to 1000 times as active as peptone.

THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN BY PEPTONE

As in the case of agar, it was essential to show that the symptoms and autopsy findings after peptone injection were due to an altered state of the blood—in short, to anaphylatoxin. If the blood became toxic as a result of the action of peptone, then transfusion of a sufficient amount to a guinea-pig should produce a typical anaphylactic shock. On the other hand, if the toxicity was due to a direct action, or to a cleavage of the peptone, the transfusion of blood containing but a small fraction of the original peptone should yield no effect.

For the experiments made with this object in view, the peptone solution was prepared as usual, the concentration being 10%, unless otherwise stated. The procedure was the same as for the corresponding agar tests (Table 65). The results agree very closely with those where agar was injected, thus showing that the same mechanism is brought into being.

Anaphylatoxin in Rats.—In Table 92 are presented the results of 8 transfusion experiments with rats which received an intravenous injection of 2 to 3 c.c. of 10% peptone solution. It will be observed that here, as in like experiments with agar, the immediate transfer of

blood from an injected rat has either no effect or but a very slight one (Nos. 1 and 2). This is an important fact, for, if the effects seen in the later tests were due to a carrying over of a lethal dose of peptone, then such transfer should certainly be more likely in the immediate tests. As it is, the effect observed was practically nil. Furthermore, it may be added that a simple calculation, based upon the amount of blood present in a rat, the amount transferred, and the amount of peptone injected, will show that the quantity of peptone which could possibly be present in the transfused blood is but one-fourth to one-half of that contained in an average lethal dose of peptone (0.06 gm. per 200 gm. guinea-pig).

As the time of reaction within the blood increased, a rapid production of poison occurred. It will be seen that this reaction time for No. 3 was but 1 minute and 18 seconds. The accompanying chart (11) is based upon the reaction times, and it will be noted that Test 5

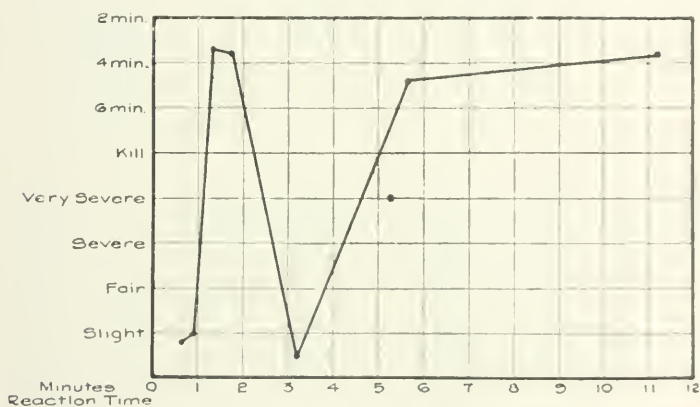


Chart 11. The in-vivo production of anaphylatoxin in rats injected with peptone (Table 92).

gave but a slight reaction which might be taken to indicate that a drop in the poison-production occurred about this time. A similar drop was obtained in the transfusion experiments with rats injected with agar (Chart 9). It was noted in connection with Test 5 that, just before the rat was operated on, the deep respiration had changed to about normal, which fact would show an individual resistance on the part of the animal, thus accounting for an apparent decrease in the poison. Further, in these tests, as in others with anaphylatoxin, it is to be remembered that the recipient may be unusually resistant. In Test 8,

where but 1 c.c. was transfused, a very severe shock was obtained. Apart from the one exception, it will be seen that the blood of the injected rats was toxic to guinea-pigs, and that this condition persisted for 11 minutes (No. 7). Control experiments, given in connection with like transfusion tests (Table 66; see also Part IX), show that as much as 4 to 5 c.c. of rat blood can be carried over into a guinea-pig without any effect, provided the transfer time is less than 1 minute.

TABLE 92
THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN RATS INJECTED WITH 10% PEPTONE

Rat		Peptone	Guinea-Pig	Blood Transfused (c.c.)	Total Time	Transfer Time	Result
No.	Weight	Intra-venous Injection (c.c.)	Weight				
1	130	2.6	175	2.0	1'10"	29"	Slight
2	148	3.0	212	"	1'15"	19"	"
3	155	"	170	"	1'44"	26"	3'21". Typical shock
4	145	"	182	"	2' 7"	28"	3'32". " "
5	152	"	196	"	3'28"	26"	Very slight
6	151	2.0	192	"	6' 2"	22"	4'58". Typical shock
7	149	"	176	"	11'39"	29"	3'31". " "
8	155	"	182	1.0	5'50"	33"	Very severe typical shock

The difference between total time and transfer time represents the reaction time within the body of the rat.

It was possible that in these experiments, the amount of blood transferred (2 c.c.) contained more than 1 lethal dose. In order to have some information on this point, a similar series of tests was made, in which 1 c.c. of blood was transfused from the injected rat to a guinea-pig. The total time for these tests was 2'29", 3'35", 3'51", 4'36", and 5'50". The effect in the first test was practically nil; in the second, slight; in the third, moderate; in the fourth, fair; while in the fifth and last one (included as No. 8 in Table 92), they were very severe. It will be seen, therefore, that the minimal lethal dose, formed in vivo is between 1 and 2 c.c. of blood. This means that in a rat of 150 gm. with about 15 c.c. of blood, there is produced somewhat more than 7.5 guinea-pig lethal doses of anaphylatoxin. This amount, however, cannot possibly account for the anaphylactic shock and death in rats injected with peptone, since it has been shown (Part V) that rats, compared with guinea-pigs, weight for weight, will tolerate 100 fatal doses of anaphylatoxin. It is possible that the peptone injection brings about a lowered cell resistance and thus renders the rat more susceptible to the anaphylatoxin; it is also possible that the cellular as well as blood plasma reacts to the peptone by poison-production.

Anaphylatoxin in Guinea-Pigs.—In the first series of attempts to demonstrate the formation of anaphylatoxin, the peptone was injected in dose of 2 gm. per kilo body weight, a 20% solution being employed. The results are to be found in Table 93. It will be seen that 7 of 12 tests ended fatally with typical shock, giving a percentage of 58. This large dose of peptone was employed for the express purpose of producing as severe a shock as possible with the object of inducing a maximal disturbance, thereby facilitating the detection of the poison.

It is deserving of note that in No. 12 with a reaction time of only 43 seconds, acute shock resulted; a like outcome was obtained in No. 10, where the reaction time was 57 seconds. This would indicate that under these conditions the poison-production occurs at great speed.

TABLE 93

THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIGS INJECTED WITH 20% PEPTONE:
2 GM. PER KILO

Donor		Peptone Intra- venous Injection (c.c.)	Time of Death	Recipient		Total Time	Transfer Time (sec.)	Result
No.	Weight			Weight	Blood Trans- fused (c.c.)			
1	500	5	3'	202	3	5' 7"	62	Very slight
1a				196	"	5' 51"	106	"
2	615	6.2	3' 40"	190	5	5' 32"	57	3' 13". Typical shock
3	650	6.5	3' 8"	205	"	4' 40"	45	Fair shock
4	505	5	3' 3"	190	"	5' 12"	47	Good shock
5	525	5.25	4' 25"	205	"	6' 54"	49	3' 46". Typical shock
6	475	4.75	3' 45"	207	"	6' 22"	57	Severe
7	510	5.1	2' 50"	198	4	6' 12"	58	6' 54". Typical shock
8	520	5.2	3' 17"	195	5	6' 32"	60	6 hr.
9	540	5.4	3' 10"	195	"	6' 27"	73	8' 5". Typical shock
10	600	6		205	"	1' 32"	35	3' 15". " "
11	550	5.5		190	"	1' 28"	40	Moderate
12	570	5.7		200	"	1' 19"	36	42". Typical shock
13*	400	4		190	"	5' 45"	40	Nil

* No. 13 is a control which received an injection of 4 c.c. of salt solution. For other controls, see Parts VIII and IX.

The other tests all show marked toxicity with the exception of No. 1, where duplicate tests of 3 c.c. gave but very slight effects. On account of the drop in blood pressure, it was not possible to make duplicate tests with larger amounts of blood; in fact, to secure 5 c.c., required an appreciable time, as will be seen from the transfer time of the tables. In Nos. 10 to 12, where the heart was exposed immediately after the injection had been made, the desired amount of blood was quickly obtained, because the effect on blood pressure had not time to manifest itself.

Inasmuch as the dose of peptone employed in the first series of tests was large and might justify the objection that the amount of blood transfused carried with it a lethal amount of peptone, a second series with but 1 gm. of peptone per kilo was made, the results of which tests are given in Table 94. Of the 13 tests in this series, 3 were acutely fatal while 1 was protracted, although the immediate effects were severe. It is to be noted that the very early transfusions in Nos. 14 to 16 gave very slight results as compared with like tests in Table 93. The percentage of fatal shocks was less than with 2 gm., namely, 31%.

TABLE 94

THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIGS INJECTED WITH 20% PEPTONE:
1 GM. PER KILO

Donor		Peptone Intra- venous Injection (c.c.)	Time of Death	Recipient		Total Time	Transfer Time	Result
No.	Weight			Weight	Blood Trans- fused (c.c.)			
14	650	3.25		192	4	1'45"	55"	Slight
15	500	2.5		200	5	1'26"	43"	"
16	490	2.45		189	"	1'31"	41"	Moderate
17	500	2.5	2'45"	200	"	4'36"	1' 3"	"
18	410	2.1	4'42"	190	4	6'35"	1'10"	2'30". Typical shock
19	500	2.5	3'	185	5	5' 5"	1'30"	Slight
20	570	2.9	?	185	"	3'28"	48"	Very severe
21	460	2.3	?	180	"	3'34"	54"	6 hr. Severe
22	550	2.8	3'	205	"	6' 3"	1'18"	Slight
23	600	3	3' 8"	180	"	6' 7"	1'	14'15". Typical shock
24	430	2.15	3'12"	205	3	7' 3"	1'18"	Fair
25	550	2.75	3'10"	180	5	6'45"	1'12"	3'40". Typical shock
26	525	2.6	4'20"	191	"	7' 8"	1' 8"	Slight

The only control test made at this time was No. 13. It had been learned previously, however, that 5 c.c. of guinea-pig blood, drawn from the heart and kept in the syringe for 2 minutes, produced little or no effect on subsequent injection (Table 117). This fact is also evident from the transfusion tests after injection of agar (Table 67), as well as in like tests after specific shock (Tables 132, 133).

In interpreting the results of the tests given in Tables 93 and 94, it is necessary to bear in mind that the individual guinea-pigs vary considerably in their resistance to anaphylatoxin. The fact that a guinea-pig gives a slight response does not prove that the poison is absent, as will be seen on reference to Table 45. The dose injected may well represent an average lethal dose, that is, such as will be fatal to one-half of the test animals. While it would be very desirable to make duplicate tests from the same heart of a treated animal, this is

quite impossible as to the 5-c.c. dose, on account of the drop in blood pressure in the donor. Any single nonfatal test must, therefore, be interpreted with considerable caution. The failure to realize this fact is in evidence in much of the literature on anaphylatoxin and anaphylaxis.

The positive results cannot be ascribed to the disturbance caused by the mere volume of the liquid injected since the control with salt solution (No. 13) was without effect. Neither can they be credited to precoagulation toxicity, since the drawn blood invariably was fluid for several minutes after the injection of the recipient. Furthermore, as mentioned, even normal guinea-pig blood can be transfused in like amount without any ill effect.

It is possible to interpret them as due to the presence of a lethal dose of peptone in the volume of blood transfused. It may be conceded that such objection is fairly valid, although Schmidt-Mülheim and also Fano claimed that the peptone rapidly disappeared from the blood and was gone in from 5 to 6 minutes after the injection. This possible interpretation is somewhat valid for the tests given in Table 93, where the donor received 2 gm. of peptone per kilo body weight.

On the assumption that all of the peptone remains in the circulation, and that the blood constitutes 7-10% of the body weight, it will be found that recipient No. 8 was given from 0.072 to 0.1 gm. of peptone in the blood transferred; for the others in Table 93, the calculated amounts thus carried over would be from 0.09 to 0.125 gm. It has been shown hitherto that a dose of from 0.06 to 0.1 gm., when given by itself is fatal to about one-half of the test animals (Tables 71, 72, 75). On the other hand, it is to be borne in mind that in a mixture of serum and this amount of peptone the action of the latter is quickly neutralized (Tables 75, 76), and it is probable that a like action may occur *in vivo*, especially with a reaction time of 5 or 6 minutes. Be that as it may, some doubt is justifiable as to whether the deaths in Table 93 were caused by anaphylatoxin or by transferred peptone.

The possible interpretation of the results mentioned can hardly hold true for the tests given in Table 94, where the donors were given 1 gm. of peptone per kilo of body weight. A similar calculation shows that recipient No. 18 could receive but 0.038 to 0.054 gm. of peptone; for the other fatal cases in this table, the amount of peptone which could possibly be transferred with the blood would be from 0.048 to 0.068 gm. This fact would seem to justify the view that these deaths were not

due to peptone carried over, but to the anaphylatoxin formed in the blood. This conclusion is given strong support by the results of Tests 14 to 16, where the transfer was made as soon as possible after the injection; in these it might be expected that the peptone would be present, if at all, in a maximal amount because of the very early transfusion. They are, therefore, essentially controls for the tests which follow. In the corresponding tests of Table 93 (Nos. 10 to 12) 2 proved fatal, and while this fact may be considered as evidence of a peptone transfer, it is conceivable that anaphylatoxin is produced more rapidly after an injection of 2 gm. than after one of half that amount.

While the results obtained in the transfusion tests with guinea-pigs are not as clean cut as those obtained with rats, still they at least make it highly probable that anaphylatoxin is produced *in vivo* when peptone is injected. It has been shown that the guinea-pig serum does not readily react with peptone *in vitro*, and this appears to be also true of the blood *in vivo*, since a relatively large dose must be given in order to produce a fatal shock. Such death may be due to the formation of but 2 or 3 lethal doses, and in that event it would be quite impossible to detect its presence by transfusion. When, however, a fair multiple lethal dose is developed, it becomes feasible to show its existence, and to arrive at some idea of the number of average lethal doses formed. Thus, in Test 23, in which 5 c.c. of blood transfused from a 600-gm. donor proved fatal, it would appear that from 8 to 12 lethal doses of poison had been produced, according as to whether the volume of blood is taken as 7 or 10% of the body weight.

Anaphylatoxin in Rabbits.—The difficulty in arriving at an unequivocal result in blood transfusion from guinea-pigs injected with peptone is accentuated in the case of the rabbit on account of the large dose which must be given in order to produce a fatal shock. This will be evident by an examination of the results of two such experiments, given in Table 95. Because of the large dose of peptone, and also because of the possibility that the blood of the rabbits might be inherently toxic, the amount of blood transfused was limited to 2 c.c. In each experiment, 2 preliminary controls were made before injecting the peptone, and these served to show an absence of inherent toxicity as to the dose employed. The blood was obtained for these tests by direct heart puncture. Thereupon, the rabbit was injected with the peptone solution, 5 gm. per kilo of body weight. In Exper. A the heart was

exposed and the blood was drawn into a heart pipet within 10 minutes after the injection, although the rabbit was not severely shocked; portions of 2 c.c. were then drawn up from the pipet and injected into Nos. 3 and 4. It should be added that the blood in the pipet remained perfectly fluid for 12 minutes, but clotted at 14 minutes (Table 80, No. 13).

Rabbit B of the other test was also tested for inherent toxicity (Nos. 5 and 6), after which it was injected with peptone, the dose being 5 gm. per kilo, and as this had but very slight effect, it was reinjected 26 minutes later with a like amount, death resulting in 3 minutes, 20 seconds (Table 80, No. 17). The heart was at once exposed and the blood was drawn up into a pipet within 6 minutes after this second injection; portions of this blood were then tested on Nos. 7 to 9. The condition of this blood is indicated in the footnote to Table 95.

TABLE 95

THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN RABBITS INJECTED WITH 30% PEPTONE: 5 GM. PER KILO

Donor		Peptone	Recipient		Blood Transfused (c.c.)	Total Time	Transfer Time	Result
No.	Weight	Intravenous Injection (c.c.)	No.	Weight				
A	1350	22.4	1	207	2		13"	Slight
			2	208	"		13"	"
			3	200	"	11'38"	1'23"	"
			4	400	"	12'19"	1'32"	10'23"
B	1930	32	5	192	"		19"	Slight
			6	195	"		27"	"
			7	192	"	8' 2"	1'47"	27'
			8	195	"	10'15"	4'	3'45"
			9	198	"	14'17"	8' 2"	Fair

Nos. 1, 2, 5, and 6 are controls preliminary to the injection. In Nos. 7 to 9 the blood was not transfused but was taken from a heart pipet; hence the apparently long transfer time which actually is the time the blood was in the pipet. The blood was perfectly fluid and remained so for 35 minutes (see Rabbit No. 17, Table 80). The same explanation holds true for the transfer time of Nos. 3 and 4.

The results, taken as they are, would appear to show that anaphylatoxin was formed under these conditions. However, the possibility that the effects observed were due to peptone transferred with the blood must not be overlooked. As in the case of the guinea-pigs, on the assumption that all of the peptone remains in the circulation, a calculation shows that the recipients may have received from 0.08 to 0.11 gm. of peptone, in other words an average lethal dose. Under the circumstances, no positive conclusion can be drawn as to the formation of

anaphylatoxin in rabbits injected with peptone. As long as it is necessary to employ Witte's peptone in such large dosage it will be difficult to settle the question. If it were true that the peptone disappears very rapidly from the circulation it might aid the solution of the problem. Perhaps experiments with a more highly toxic proteose, one which will kill in dose of 1 gm. or less per kilo, will give decisive results.

SUMMARY

The symptoms and findings in guinea-pigs injected with peptone are the same as those produced by anaphylatoxin, by agar, or by specific anaphylactic shock. A marked exophthalmos may be present, a symptom especially marked in rabbits.

Guinea-pigs show the same individual variation to peptone as has been demonstrated for anaphylatoxin and for agar; this variation extends to the several symptoms and findings.

Peptone in a dose of 0.75 gm. per kilo will usually kill guinea-pigs, but occasional failure has been noted even with 1 gm. per kilo. On the other hand, 0.3 gm. per kilo represents an average lethal dose, since it is fatal to about one-half of the test animals.

The speed of injection is a factor in the results obtained.

After a nonfatal injection, guinea-pigs show the same immunity or tolerance as has been observed in dogs. Repeated injections of a sublethal dose, at intervals of 15 minutes, result in death. The tolerance is often broken down by the injection of peptone solutions diluted with from 6 to 9 parts of salt solution or distilled water.

An hypertonic salt, confirming Ritz, may protect against an otherwise fatal dose of peptone. An even more marked antagonism is shown by sodium carbonate, which also acts against anaphylatoxin. This fact supplies a fundamental basis for the preventive and therapeutic action of alkali as to blood disturbances. Normal serum also possesses a decided antagonistic action.

Injections of peptone markedly affect the coagulation time of guinea-pig blood; the larger the dose, the more pronounced the effect.

The injection of peptone into white rats results in symptoms and findings identical with those caused by anaphylatoxin and by agar. The coagulation time of the blood may be retarded for an appreciable period, even up to 22 minutes.

The least lethal dose for the rat was found to be 2 gm. per kilo, an amount 7 times larger than that needed for guinea-pigs.

In rabbits, very large doses of peptone must be employed; perfectly typical anaphylactic shock and findings may be obtained. In addition to the usual symptoms such as dyspnea, spasms, convulsions, a striking exophthalmos is to be noted, also, a great drop in blood pressure, and a marked decrease in coagulation time. Considerable variation in the resistance of rabbits, like that in rats and guinea-pigs, was found.

The least amount of peptone which was fatal to the rabbit was 3 gm. per kilo, but some may withstand even 5 gm. per kilo. The rabbit is, therefore, more resistant than the rat. It is a striking fact that the rabbit is considerably more resistant than the guinea-pig to agar, peptone, anaphylatoxin, and to specific anaphylatoxic shock.

The rabbit shows an immunity or tolerance to repeated injections of peptone, the same as the dog or guinea-pig.

The change in the coagulability of the rabbit blood occurs immediately after peptone injection, even in nonfatal cases. The coagulation may be completely retarded for 35 minutes. At times the process does not go beyond the formation of a small clot (+), which soon shrinks and may be overlooked (fibrinolysis of Dastre).

Peptone is considerably less toxic than agar. The ratio expressing the relation of the least toxic dose of agar to that of peptone is 1:33 for the guinea-pig; 1:74 for the rat; and 1:188 for the rabbit.

Rat serum on treatment with peptone rapidly yields anaphylatoxin. A peptone suspension is more active than a perfectly clear solution. The fully dissolved peptone does not toxify rat serum as easily, nor does it yield as high a degree of toxicity as do the agar and trypanosome suspensions. Extreme dispersion is, therefore unfavorable for the reagent.

Rat serum in dose of 2 c.c. can be toxified in less than a minute.

With peptone, rat serum gives the best results when used as soon as prepared; even then variations due to the individual peculiarities of the animal occur. In the case of agar, a vastly more active reagent, these conditions are rarely in evidence.

Experiments made with guinea-pig serum, treated with peptone, indicate that here also anaphylatoxin is produced. The weak action of the peptone, and the feeble reactivity of the serum, as compared with that of the rat, render the demonstration of the poison more difficult. This is still more true when rabbit serum is tested with peptone.

A comparison of the effective agar-serum ratio with that of peptone-serum shows that with regard to rat serum, agar is 500 times

more active than peptone; with regard to guinea-pig serum, it is 200 to 1000 times as active.

The transfusion of the blood of rats injected with peptone demonstrates that anaphylatoxin is formed *in vivo*. In the rat somewhat more than 7.5 guinea-pig lethal doses were found.

Similar transfusions in the case of guinea-pigs likewise showed that anaphylatoxin was produced. In rabbits, on account of the large dose of peptone necessarily injected, the results were not as decisive.

Peptone itself is not toxic, but when brought into contact with a reactive serum or plasma it induces the change which results in anaphylatoxin-production.

VIII. THE PRIMARY TOXICITY OF NORMAL SERUM

PAUL HENRY DEKRUIF

SYNOPSIS

INTRODUCTION

HISTORICAL SUMMARY

EXPERIMENTAL PART

THE TOXICITY OF NORMAL RABBIT BLOOD AND SERUM

THE TOXICITY OF FRESH UNDEFIBRINATED RABBIT BLOOD

RAPID DECREASE OF TOXICITY OF FRESH RABBIT BLOOD

INDUCING POWER OF FRESH UNDEFIBRINATED RABBIT BLOOD

INDUCING POWER OF RABBIT SERUM

APPARENT VARIATION IN TOXICITY OF NORMAL RABBIT
SERUM

EFFECTS PRODUCED BY NORMAL RABBIT SERUM

RELATION OF DOSE TO PICTURE AT AUTOPSY

ANTAGONISTIC ACTION OF SODIUM OXALATE

EFFECT ON TOXICITY OF SPONTANEOUS CLOTTING; MINIMAL
CONTACT WITH CLOT; CONTACT FOR 30
MINUTES; CONTACT FOR 20 MINUTES

EFFECT ON TOXICITY; BEAD DEFIBRINATION AT ROOM
TEMPERATURE; AT 40 C.; CHILLED BEAD DEFIBRINATION

COMPARATIVE TOXICITY: ROD AND BEAD DEFIBRINATION

EFFECT ON TOXICITY: CHILLED ROD DEFIBRINATION;
RAPID CLOTTING WITH CHILLED BEADS

COMPARATIVE TOXICITY: BEAD DEFIBRINATION AT 40
AND AT 0 C.

TOXICITY OF SERUM KEPT IN CONTACT WITH CLOT:
FREED FROM CONTACT WITH CLOT

COMPARATIVE STABILITY OF SERUM TOXICITY AT 37 C.
AND AT ROOM TEMPERATURE

TOXICITY OF DEFIBRINATED BLOOD, OBTAINED WITH
HEATED BEADS

THE TOXICITY OF NORMAL GUINEA-PIG BLOOD AND SERUM

THE TOXICITY OF FRESH UNDEFIBRINATED GUINEA-
PIG BLOOD

TOXICITY OF NORMAL GUINEA-PIG SERUM: ROD DEFIBRINATION;
AFTER SPONTANEOUS CLOTTING; BEAD
DEFIBRINATION

TOXICITY OF GUINEA-PIG BLOOD; ROD DEFIBRINATION;
CHILLED BEAD DEFIBRINATION

THE TOXICITY OF NORMAL RAT SERUM

SUMMARY

One finds it to be the impression of most workers who have concerned themselves with the study of anaphylatoxin, that the serum of the guinea-pig is harmless when introduced into the circulation of an

animal of the same species. From all sides, evidence is presented that the serum of the guinea-pig, in itself harmless, becomes toxic after appropriate treatment.

Stühmer,¹ while noting the work of other writers on the toxicity of homologous serum for rabbit and guinea-pig, thought that this toxic condition persists for but a short time after drawing of the blood. Several workers, among whom is Haren,² believed that normal guinea-pig serum is innocuous for like animals, as does also Tchernoroutzky.³ The latter gives from 2 to 3 c.c. per 100 gm. as the fatal dose of rabbit serum anaphylatoxin for guinea-pigs, although smaller amounts of normal rabbit serum are frequently lethal for these animals.

It would seem, then, that a study of the normal sera, unchanged by the action of alien substances, would be of considerable value. We wish to emphasize, in the beginning, that we are aware that this conception of nontoxicity is not prevalent in regard to all heterologous sera. Mosso long ago described the toxicity of eel serum. Doerr and Raubitschek,⁴ state that the serum of the eel is fatal for the guinea-pig in doses of 0.01 c.c. What is more, dog serum is toxic for the guinea-pig in doses of from 1.5 to 2 c.c. Beef, sheep, and goat sera have been shown to be fatal in somewhat similar amounts, while normal, human serum killed small guinea-pigs in doses of from 0.5 to 0.75 c.c.

Before proceeding to the experimental part of our work, it will be well to give a brief account of the various ideas of the cause of normal serum toxicity.

HISTORICAL SUMMARY

* The earliest record of the toxicity of normal blood is to be found in the report of the commission of the Royal Philosophical Society, published in 1666. The commission, appointed to inquire into the cause of transfusion accidents, concluded that these were due to the intravascular clotting of the transfused blood. The early transfusion work was done usually with heterologous blood. Other explanations, besides that of thrombosis, were brought forward. Magnani,⁵ who injected sheep blood into dogs, concluded that the fatal result was due to the use of a too great volume. Bischoff⁶ thought that the venosity of the blood was the cause of its toxicity, and Brown-Sequard⁷ held a like opinion.

The work of Landois⁸ must be looked upon as opening a new era in the investigation of normal blood toxicity. He was the first to employ serum.

¹ Deutsch. med. Wehnschr., 1912, 38, p. 2123.

² Ztschr. f. Immunitätsf., 1913, 20, p. 673.

³ Compt. rend. Soc. de biol., 1913, 74, p. 1213.

⁴ Berl. klin. Wehnschr., 1908, 45, p. 1525.

⁵ Paul Scheel: Die Transfusion des Blutes, usw. Quoted by Coca.

⁶ Müller's Archiv, 1835, p. 347; Schmidt's Jahrb., 1836, 12, p. 16.

⁷ Leçons sur les phénomènes physiques de la vie, 1842, t. 4.

⁸ Die Transfusion des Blutes, Leipzig, 1875.

rather than whole blood, thus eliminating one complicating factor, the foreign, red blood cells. He used rabbits and dogs as recipients, and tested the effect of a large number of heterologous sera on these animals. Experiments in the test tube were made for the first time by Landois who found that the sera which were toxic for the rabbit also dissolved or flocked the erythrocytes. From these experiments, it was concluded that the agglutinating sera, when injected into the veins of the rabbit, cause intravascular flocking of the recipient's red blood cells and consequently capillary blocking, resulting in death. The hemolyzing sera, on the other hand, were believed to kill by liberating thromboplastic substances from the erythrocytes, with resulting intravascular clotting, plugging of the lung and brain capillaries, and death.

Until quite recently, all explanations have confined themselves to the ideas of thrombosis, of hemagglutination, or of hemolysis. Capillary plugging, resulting from small clots or from flocked erythrocytes, is by far the most common explanation of the toxicity of normal serum. We shall glance at the evidence for and against these theories, considering, first, thrombosis.

Köhler⁹ injected a rabbit intravenously, with its own blood previously defibrinated, and produced acute symptoms and death. He found thrombi in the heart and large vessels, at autopsy, and concluded that the injected blood was toxic because of its high fibrin-ferment content. Schenk¹⁰ and Moldovan¹¹ also think that the toxicity of homologous, as well as of heterologous blood, is due to its thromboplastic activity. We shall have occasion to discuss the work of Moldovan in another connection.

Boggs¹² has shown that the thromboplastic activity of a fresh serum for plasma in the test tube does not run parallel to its action when introduced into the body. By the addition of a little alkali to fresh rabbit serum, he was able greatly to increase its thromboplastic action. Such serum, however, injected into a rabbit, did not change the coagulation time of its blood. Morawitz¹³ also opposed the idea of fibrin-ferment as being the cause of transfusion accidents. He gives no protocols and merely quotes v. Jürgensen's¹⁴ work. This author found that clotting occurred only when the injected blood was so fresh that it was still warm.

Loeb, Strickler, and Tuttle¹⁵ refer the toxicity of dog blood for rabbits to its ability to hemolyse the red cells of this animal. The hemolysis that occurs when the dog-serum is injected is believed to liberate clot-accelerating substances. These clots lodge in the lung capillaries and cause death. This theory is based on the fact that hirudin protects against this serum. But it is to be noted that this protection is only partial; that when a dose twice as large as one that would kill without hirudin was given, the injected animal would succumb, but without any visible intravascular clotting. This weakens the hypothesis of these investigators. Furthermore, Loeb, Strickler, and Tuttle admit that the thrombi in the heart and pulmonary vessels are essentially agonal ones; that they occur only as the animal is dying. The authors state that the capillary clots may occur before this. This assertion awaits demonstration. In the light of the present evidence, there is as much possibility of

⁹ Inaug. Diss., Dorpat, 1877.

¹⁰ München, med. Wehnschr., 1910, 57, p. 903.

¹¹ Deutsch. med. Wehnschr., 1910, 36, p. 2422.

¹² Deutsch. Arch. f. klin. Med., 1904, 79, p. 539.

¹³ München, med. Wehnschr., 1907, 54, p. 767.

¹⁴ Von Ziemssen's Handbuch d. Allgemein. Therapie, 1883, 1, p. 149.

¹⁵ Virchows Arch. f. path. Anat., 1910, 201, p. 5.

thrombosis being a phenomenon, concomitant with the intoxication, as of its being a cause of the latter.

Landois⁸ was the first to think of the hemagglutinating power of a serum as being a cause of its toxicity. Battelli⁹ has brought forward some interesting work in this connection. The washed erythrocytes of various species were injected into rabbits, which stood well the injection of dog, cat, and beef, red cells, but succumbed to those of the pig, sheep, and rat. Tests, *in vitro*, showed that rabbit serum hemolysed the erythrocytes of the latter, while it left intact those of the former group of animals. In another paper, Battelli¹⁷ showed that, while beef and dog red blood cells are harmless to normal rabbits, they were toxic to those specifically immunized. He also showed that the accident resulting from injection was not due to products liberated during hemolysis. On the other hand, the red cell stromata might be substituted for the intact cells without altering the effect described above. While Battelli's conclusion is that the cause of the toxicity of the cells is due to their flocking, by specific hemagglutinins of the rabbit serum, it is more likely that we have here the first clean-cut demonstration of red-blood-cell anaphylaxis.

Coca¹⁸ injected washed heterologous erythrocytes into rabbits, and he, too, concludes that the resulting shock is due to an agglutination of these cells in the capillaries. He does not believe that this flocking is due to the presence of a specific agglutinin only, but comes about through the influence of some substances residing probably in the capillary walls. Loeb, Strickler and Tuttle¹⁹ think that the toxicity of beef serum for rabbits is due to its hemagglutinating properties.

The precise researches of Zinsser²⁰ have undermined largely the validity of the hemagglutination hypothesis. This author, testing the effect of goat serum upon rabbits, showed that the red-cell-flocking properties and toxicity do not run a parallel course. Fresh goat serum flocks rabbit erythrocyte energetically. It is also very toxic for the rabbit. But while heating at 56° for 20 minutes destroys its toxicity, it leaves the agglutinating power, intact. The same relation may be shown by keeping the serum for a few days.

Of the three hypotheses mentioned above, it remains to discuss that of hemolysis. Landois' work was, also, in this instance the starting point. He showed that a serum that will dissolve red cells of a rabbit will also kill the animal. It must be emphasized here that such a demonstration does not correlate the two properties. Pfeiffer²⁰ showed that normal heterologous sera caused infiltrations when injected subcutaneously into guinea-pigs. He concluded that the effect was due to the hemolysin in the heterologous serum. Heating at 56°, according to Pfeiffer, destroyed the hemolytic, but not the toxic action. In his opinion, the hemolysin of the inactive serum was reactivated in the body of the guinea-pig by its own alexin. On the contrary, Uhlenhuth and Haendel²¹ have shown that guinea-pig alexin does not reactivate the toxicity of inactive beef serum, although it restores, to a large extent, its hemolytic power.

The last mentioned investigators studied particularly the necrotizing action of foreign sera when injected subcutaneously into guinea-pigs. While the experiment mentioned above refutes in a large measure Pfeiffer's claims,

¹⁶ *Compt. rend. Soc. de biol.*, 1904, 56, p. 1041.

¹⁷ *Compt. rend. Soc. de biol.*, 1904, 57, p. 17.

¹⁸ *Virchows Archiv f. path. Anat.*, 1909, 196, p. 92.

¹⁹ *Jour. Exp. Med.*, 1911, 14, p. 25.

²⁰ *Ztschr. f. Hyg. u. Infektionskr.*, 1905, 51, p. 183.

²¹ *Ztschr. f. Immunitätsf.*, 1909, 3, p. 284.

another observation does this even better. The serum of shoats and of adult pigs dissolves the erythrocytes of the guinea-pig, but only that of the adult pig causes infiltration and necrosis when injected subcutaneously.

As early as 1904, Camus and Gley²² made an important contribution to the solution of this question. These workers showed that the red blood cells of the marmot are very resistant to the action of eel serum. It requires one-twentieth to one-fiftieth dilution in physiologic salt solution to produce hemolysis in from 15 to 20 hours. Dog serum dissolves the same cells in an approximately like titer (one-twentieth to one-fortieth). But while a dose of eel serum of 0.02 c.c. per kilo kills the marmot, dog serum is tolerated by this animal in doses of from 5 to 10 c.c. per kilo. Further, while the erythrocytes of the marmot are susceptible only to high concentrations of eel serum, yet the killing dose is six times as small for the marmot as for the rabbit, whose red cells are dissolved in 1/15,000 dilution of this serum. This experiment dissociates very well the hemolytic and toxic action of eel serum. If one had here to rely only on the analogy of heat destruction, the opposite conclusion would have been reached, since heating at 56° destroys the hemolytic power and, also, to a large extent, the toxicity.

Zinsser¹⁹ found that goat serum, upon inactivation, loses both its toxic properties for the rabbit, and its power to dissolve the red cells of this animal. Guinea-pig serum (alexin) reactivates the hemolytic property but not the toxic power. Furthermore, active goat serum, which has been first treated by rabbit cells so that the sensitizer and alexin are completely removed, retains its original toxicity. The objection might be raised that this toxicity may be due to products resulting from the hemolysis occurring during this treatment. Zinsser discounted this by control injection of equivalent amounts of the products of laked rabbit erythrocytes. Such extracts were shown to be harmless. Battelli¹⁶ and others have demonstrated that the products resulting from the laking of red cells are harmless, while Coca has shown that hemolysis can occur, *in vivo*, without a fatal result.

In 1910, the toxicity of normal blood was correlated for the first time with the phenomenon of anaphylaxis by Doerr.²³ According to Doerr, both the symptom complex and the autopsy findings, after normal serum injections, correspond closely to those of anaphylaxis. Doerr and Moldovan²⁴ observed while studying the effects of fresh normal beef serum on guinea-pigs, that a syndrome, identical with that of anaphylactic shock, occurs; that atropin protects; and that anti-anaphylaxis can be demonstrated by preliminary small injections.

Zinsser,¹⁹ observing the autopsy findings on rabbits which had been killed by intravenous injections of fresh goat serum, arrived at a somewhat different conclusion. Blood coagulation was delayed, but there was no Auer-Lewis phenomenon, which Doerr and Moldovan had found present in the work described above, nor could Zinsser protect by means of atropin. He also failed to protect by preliminary small injections. On the other hand, the characteristic temperature depression, blood pressure drop, and alexin disappearance did occur. It is well to remember that these workers were testing the toxic effects of foreign sera on different species and that their findings are not, therefore, strictly comparable.

²² Compt. rend. Acad. d. sc., 1905, 140, p. 1717. Arch. internat. de pharmaco., 1898, 5, p. 261.

²³ Ztschr. f. Immunitätsf., R. 1910, 2, p. 117.

²⁴ Ztschr. f. Immunitätsf., 1910, 7, p. 223.

Doerr²⁵ believed that the toxicity of normal blood, especially that of homologous blood, occurs according to a mechanism similar to that which causes the toxification of a serum by the introduction of bacteria or inert substances, such as kaolin or colloidal silica. Doerr's interpretation is briefly this: Blood or blood serum contains preformed toxic substances held in abeyance by hindering bodies. The removal of these hindering bodies by some absorbant disturbs the colloidal-equilibrium of the serum, with a resulting liberation of the toxic substances. Thus, when clotting occurs, the precipitating fibrin acts as the disturbing element. According to Doerr, the unmasking of the toxin during clotting is transient, since the blood rapidly becomes harmless. Doerr does not interpret Moldovan's¹¹ findings on the toxicity of the blood of rabbits for homologous animals on a basis of thromboplastic activity. Further, in investigations of which he does not give the protocols, Doerr²⁵ states that it is not necessary to defibrinate with glass pearls to make a blood toxic, but that blood which clots slowly in paraffined vessels also undergoes this temporary change. Not only the whole blood, but also the serum, and even the blood cells are toxic shortly after coagulation sets in.

Slatinéano and Ciuca²⁶ showed that fresh guinea-pig serum, injected three-quarters of an hour after the blood had been drawn, is toxic in large doses to homologous animals; that this toxicity drops off within 48 hours like that of heterologous serum; and that the toxic power lost by aging can be reactivated by the introduction of a foreign colloid, such as semi-solid agar gel (Bordet²⁷). These workers agree with Doerr in that they look upon the toxicity after clotting and the subsequent reactivation by agar as due to identical mechanisms. The work of W. H. Schultz²⁸ is suggestive in this connection. He found that freshly drawn homologous blood applied to normal, smooth muscle produced no response in such muscles until clotting set in. When this became visible, a violent contraction of the muscle resulted. This would indicate that disturbances resulting in the blood toxification do not set in until clotting occurs. Schultz apparently did not investigate the effect of aging on the power of a homologous serum to produce contractility.

Zadik,²⁹ on incubating guinea-pig serum, found that this serum in a dose of 4 c.c., at first harmless, becomes toxic for homologous animals in from 2 to 3 days, and that this toxicity disappears in from 4 to 6 days. In the majority of researches upon anaphylatoxin, the investigators state that guinea-pig serum is harmless and only becomes toxic after one incubates the serum with bacteria or with a foreign colloid. From the work referred to above, it is seen that this is not the case, and that the toxicity induced is only an intensification of a state of affairs already present in the untreated serum.

The serum of a rabbit furnishes a still better reagent for the demonstration of this relationship. Here, the normal toxicity for the guinea-pig is more intense than is that of homologous serum; further, the drop in toxicity after defibrination is neither as rapid nor as great. Moldovan¹¹ states that freshly defibrinated rabbit blood is toxic for guinea-pigs, but he does not mention in what dose it is fatal. Gräfenberg and Thies³⁰ state that the serum of pregnant rabbits is fatal to guinea-pigs in doses of about 2 c.c. per 100 gm. Immediately

²⁵ Wien. klin. Wchnschr., 1912, 25, p. 339.

²⁶ Compt. rend. Soc. de biol., 1913, 74, p. 631.

²⁷ Compt. rend. Soc. de biol., 1913, 74, p. 225.

²⁸ Physiological Studies in Anaphylaxis, Bull. Hyg. Lab., U. S. P. H. S., 1912, 80.

²⁹ Folia serol., 1911, 7, p. 865.

³⁰ Ztschr. f. Immunitätsf., 1911, 9, p. 749.

after the birth of the young, the toxicity rises to 1 c.c. per 100 gm. No mention is made of the toxicity of normal, male rabbit serum for guinea-pigs. These workers do not agree with Moldovan¹¹ and with Schenk¹⁰ that death is due to thromboplastic substances. They state that the symptom complex and the autopsy findings are identical with those of anaphylaxis. Doerr and Weinfurter²¹ found normal, rabbit serum to be fatal to guinea-pigs in doses of about 3 c.c. per 100 gm. The toxicity of rabbit serum could be increased about 3 times, by bleeding the rabbit frequently over a period of 100 days or more. It is the conclusion of Doerr and Weinfurter, too, that the symptoms and the findings, at section, resemble those of anaphylactic shock.

Mita and Ito²² tested the toxicity of serum from a large number of rabbits. Once they succeeded in obtaining a very toxic serum, the lethal dose being 0.5 c.c. per 100 gm. But in the majority of instances, the fatal dose ranged from 1.5 to 2 c.c. per 100 gm. These workers claim to show a drop in toxicity after the blood has remained for some time at room temperature. This falling off is supposed to occur in from 1½ to 4 hours after bleeding. A study of their protocols does not show this toxicity decrease in a very convincing manner. In fully one-third of the cases, no drop at all can be detected. Inactivation, according to Mita and Ito, stabilizes the toxicity of rabbit serum. These workers draw no conclusion as to the reason for this toxicity, and they do not indicate that they have performed autopsies on the animals killed.

Little stress is laid by the majority of workers on the relationship between the toxicity of heterologous and homologous blood and the method of defibrination. Moldovan¹¹ does mention such a relationship. He states that rabbit blood, which is shaken vigorously for 5 minutes with porcelain beads, is more toxic than blood defibrinated for a shorter period of time. He also makes the observation that rabbit blood is not toxic for homologous animals, when it is injected before clotting. Still earlier, Studzinski²³ mentions a failure to make a homologous blood toxic for dogs, when such blood is defibrinated with a broom-like contrivance. On the other hand, he finds that blood defibrinated with beads is very poisonous. In almost all cases, those investigating this problem are vague as to the details of the technique used in preparing the serum under test. We believe that the toxicity of normal guinea-pig and rabbit sera is largely influenced by the way in which the process of coagulation occurs, a fact which we propose to demonstrate in the experimental part which follows.

EXPERIMENTAL PART

THE TOXICITY OF NORMAL RABBIT BLOOD AND SERUM

The Toxicity of Fresh, Undefibrinated Rabbit Blood.—We propose to take up, first, the toxicity of normal rabbit whole blood and serum. Its relation to coagulation will be particularly studied in order to gain a basis for a sound interpretation of its nature and origin. It is important to know whether the circulating blood of normal animals is toxic, or whether it becomes so as a result of extravascular changes. At the outset of this study, it was assumed that a normal blood or serum, such

²¹ Centralbl. f. Bakteriöl., I Orig., 1913, 67, p. 92.

²² Ztschr. f. Immunitätsf., 1913, 17, p. 586.

²³ Zentralbl. f. Physiol., 1909, 23, p. 755.

as that of the rabbit, possessed reasonably constant qualities; indeed, it was believed that the blood of normal animals was perforce nontoxic, since it seemed difficult to understand how an animal could be seemingly in perfect condition, and yet, a carrier of poison. As the work broadened, it was realized that different species of animals showed extreme variation as to their susceptibility to anaphylatoxin, and that even animals of the same species exhibited considerable variation. These facts must be borne in mind in connection with the observations to be presented.

In the work which follows, strictly normal rabbits were used; they were all large or medium sized. In general, they were taken from the pen in the morning, before the time of feeding. By this procedure a clear, non-lipoidal serum was invariably obtained. The recipients were usually guinea-pigs, varying in weight from 170 to 210 gm.; to a less extent, white rats were also used. The latter, as in the case of anaphylatoxin work in general, proved to be of extreme value. The criterion of death, in the guinea-pig, was the nasal reflex, which occurs in practically all cases of shock which terminate fatally.

In order to determine how soon the blood of a normal rabbit, after its withdrawal from the body, became toxic, recourse was had to transfusion experiments, such as are given in Table 96. For these tests, the blood was drawn from the heart, by direct puncture of the thoracic wall, into a syringe, previously rinsed in 0.85% salt solution. It was then kept in the syringe for a given time (column headed, "interval"), and injected into the exposed jugular vein of a guinea-pig. Each test represents a separate puncture; the time required to draw up the blood into the syringe ranged from 7 to 20 seconds, while that for the injection was from 3 to 15 seconds. One rabbit served for tests Nos. 1 to 6, inclusive; another for test No. 7, and still another for test No. 8.

It will be seen from Series A, Table 96, that in the dose employed, the rabbit blood, up to about 3 minutes after being drawn, was but slightly toxic; when kept in the syringe for 3 minutes it caused a severe reaction, while that held for $3\frac{1}{4}$ minutes produced fatal shock. The symptom complex resembled closely that of true anaphylactic shock. Autopsy performed 3 minutes after death showed the Auer-Lewis phenomenon, persistence of heart beat, and fluid blood. Very small clots were present in the right heart of Nos. 5, 6, and 7.

At the stage where the injection of 3 c.c. resulted fatally, no visible clot could be detected in the syringe. Although the needle was very

fine, the blood passed through easily during the injection. It is very likely, however, that a change in the aggregation of fibrinogen had already occurred. It is not necessary for such change to be visible to the eye. A microscopic or submicroscopic alteration in the aggregation might have accompanied the disturbance which induced the toxicity noted.

Very large amounts of undefibrinated blood can be injected without injury, provided the transfusion be sufficiently rapid. Thus, with a transfer time of 30 seconds, 10 c.c. of the blood of a rabbit were transfused into guinea-pig No. 8 with practically no ill effect. This and similar tests seemed to show that the blood of a normal rabbit

TABLE 96

TRANSFUSION OF NORMAL RABBIT BLOOD TO THE GUINEA-PIG (A); AND TO THE RAT (B)

Series	Recipient			Interval* (min.)	Total Time	Result
	No.	Weight	Blood Trans- ferred (c.c.)			
A Guinea-Pigs†	1	210	3	½	50"	Nil
	2	211	"	1	1'14"	Moderate
	3	207	"	2	2'16"	Slight
	4	180	"	3	3'21"	Severe
	5	209	"	3½	3'39"	4'53". Typical shock
	6	220	"	"	3'50"	3'17". " "
	7	173	"	"	4' 7"	" " "
	8	210	10	"	30"	Slight
B Rats	1	125	3	½	1'15"	Nil
	2	120	"	1	1'40"	"
	3	130	"	2	2'39"	Slight
	4	140	"	3¼	3'57"	2'38"

* This represents the time from the end of bleeding to the start of injection, full time in syringe. Total time is the period from the start of drawing the blood to the end of the injection of guinea-pig or rat.

† See Tables 69, 97, and 98.

was nontoxic, provided it was rapidly transferred. This conclusion, however, is not strictly true, for it has been shown in connection with Table 69 that it is possible for apparently normal rabbits to have a blood which possesses a high initial toxicity. The transfusion of 2 c.c. of heart blood, even at the maximal speed of less than 20 seconds, may cause acute death in guinea-pigs. Of 21 rabbits thus tested, 4 were found to have such toxic blood; the records of 3 of these are to be found in Table 69, and that of the fourth is given in Table 97, where it will be noted that even 1 c.c. of the circulating blood, was acutely fatal though the transfer time was but 10 seconds.

The variation in the toxicity of normal rabbit blood becomes readily understood when it is recalled that this animal is very resistant to anaphylatoxin (Part V). It may tolerate a considerable number of guinea-pig lethal doses without any ill effect; hence, if through some unknown cause, 50 or 100, or even 200 of such doses are generated within the body, the rabbit may carry them without any apparent change in its general condition. The normal rabbit, therefore, is subject to great variation as to the inherent toxicity of the circulating blood, and it follows that the primary toxicity of its serum may be expected to show a like variation. The chief reason for this change in the quality of the blood is probably to be found in the food. Thus, of 2 rabbits, kept side by side for 3 months, one on an exclusive diet of cabbage, and the other on carrots, the former gave a heart blood which caused acute death in dose of 5 c.c., the transfer time being 22 seconds, whereas 3 c.c. were without effect. The rabbit fed on carrots had a less toxic blood, since 7 c.c. produced but a very slight effect, the total time of transfusion being 43 seconds; while 10 c.c., with a transfer time of 1'10", proved fatal in 6 minutes.

In Series B of Table 96 are given transfusion tests from a single rabbit to white rats. It will be observed that this species responds to pre-clot blood in the same way as the guinea-pig. In dose of 3 c.c., the rabbit's blood was without effect until it had been held in the syringe for 3 $\frac{1}{4}$ minutes when it resulted in acute death. The autopsy was typical of anaphylatoxin except for the presence of a slight clot in the heart. The symptoms in rats injected with preclot blood are of the usual anaphylatoxin type: dyspnea, spasms, convulsions and marked drop in blood pressure. It is rather striking that the rat should be about as susceptible to precoagulation toxicity as the guinea-pig, although, weight for weight, the rat can tolerate 100 times as much anaphylatoxin as the latter. It would seem from this as if the toxicity of the blood in the pre-coagulation stage was not due entirely to anaphylatoxin; it is necessary to conceive of a labilized state which induces the production of this poison in the blood of the recipient. That such is the case will appear from the transfusion experiments given in Table 100.

Rapid Decrease in Toxicity of Fresh Rabbit Blood.—It has just been shown that the blood of a normal rabbit may suddenly acquire toxic properties in the period immediately preceding visible coagulation. It is pertinent to inquire whether this poisonous quality persists

in the defibrinated blood after coagulation, and is maintained in the free serum. The effect of various conditions on the production and maintenance of toxicity will be considered later on, but at this point it is desirable to bring out the main fact that a decrease occurs in the toxicity of the serum as compared with that of the blood or its plasma.

It will be seen from test No. 3, Table 97, that even 1 c.c. of the heart blood of a normal rabbit, under maximal speed of transfusion of 10 seconds, was acutely fatal. On the 10% basis, the rabbit carried 221 c.c. of blood, or that number of guinea-pig lethal doses; expressed in other words, it carried per 200 gm. of body-weight, 20 of such lethal doses. In view of this high toxicity of the whole blood, it was decided to test that of its plasma, and of its serum.

TABLE 97
COMPARATIVE TOXICITY OF A NORMAL RABBIT'S HEART-BLOOD, PLASMA, AND SERUM

Donor		Recipient		Intravenous Injection (c.c.)	Total Time	Result
No.	Weight	No.	Weight			
15	2212	1	195	2 blood	16"	2'40"
		2	208	" "	15"	2'30"
		3	200	1 "	10"	3'
		4	197	0.5 "	14"	Very slight
		5	202	0.6 plasma	6'	6'30"
		6	201	1.0 "	6'37"	4'43"
		7	215	0.6 serum	12'25"	Nil
		8	206	1.0 "	14'35"	3'50"
		9	215	" "	"	5'15"
		9a	220	" "	"	6'40"
		10	200	" "	40'	48'
		10a	205	" "	"	Very depressed
		11	207	2.0 "	56'	3'20"

The total time represents that from start of drawing blood until the end of injection. The blood for Nos. 1 to 4 was drawn each time by heart puncture, and at once injected.

The rabbit was, therefore, bled into a carotid pipet (65 seconds) and a portion of the blood was centrifugated at once at 3000 revolutions for 3 minutes; the resulting clear plasma was then injected into Nos. 5 and 6. It is to be noted that this plasma killed in dose of 0.6 c.c., an amount which corresponds to 1 c.c. of blood, previously found to be fatal (No. 3). This injection was made 6 minutes after the start of bleeding; the plasma was perfectly fluid, and an unused portion did not clot until 3 minutes after the injection of Nos. 5 and 6. In this case the observed toxicity of the plasma cannot, therefore, be ascribed to pre-coagulation change, but rather indicates the presence

in the blood, at the moment of withdrawal, of a preformed poison, or, what is more likely, a substance which induces poison production in the recipient.

A second portion of the blood was at once whipped with the glass rod for 3 minutes, at the end of which time a heavy clot began to form. This blood was then centrifugated at 3000 revolutions for 3 minutes. It was intended to test this serum at once, that is, within 6 minutes after the start of bleeding, in order to compare the toxicity with that of the plasma, but this was not feasible and the serum was kept 5 minutes before being tested. It will be seen from test No. 7 that this serum in dose of 0.6 c.c. had no effect, while 1 c.c. was fatal. Apparently, even in this short time, a slight decrease in toxicity as compared with that of the plasma had occurred.

TABLE 98
COMPARATIVE TOXICITY OF A NORMAL RABBIT'S HEART-BLOOD AND SERUM

Donor		Recipient		Intravenous Injection (c.c.)	Total Time*	Result
No.	Weight	No.	Weight			
10	2275	1	200	2 blood	15"	5'40"
		2	205	" "	"	5'
		3	205	" "	20"	3'10"
		4	210	1 serum	47'	Very slight
		5	205	1.5 "	52'	Slight
		6	189	1.75 "	55'	Very slight
		7	180	2 "	1 hr. 4'	Good shock
		8	215	3 "	1 " 12'	Slight
		9	190	3.5 "	1 " 36'	"
		10	205	4 "	1 " 46'	Moderate
		11	195	5 "	1 " 55'	1 hr. 30 min.
		12	207	7 "	2 " 19'	47'

* The total time is that from start of drawing blood until the end of injection.

For Tests Nos. 1 to 3, the blood was drawn, each time by heart puncture; the rabbit was then bled into a carotid pipet, the blood subjected to rod defibrination, then centrifugated at 8000 r.p.m. for 5 minutes. The time for Nos. 4 to 12 is counted from the start of bleeding.

After the removal of the partially defibrinated blood for the tests just given, the remainder was whipped till completely defibrinated (10 minutes), then centrifugated and tested, in pairs, at 20 minutes from the start of bleeding. In 1 c.c. dose, this serum was acutely fatal, as was the serum used in test No. 8. Another pair of tests were made 20 minutes later, the serum being kept in the room; the animals showed great depression, but no spasms or other acute symptoms; one recovered, while the other died after a long delay. Consequently, this serum appears to have lost about half of its original toxicity in the intervening

short time. The possibility that the two test animals were highly resistant must be borne in mind.

A somewhat similar experiment to that given above will be found in Table 69; the blood of the rabbit was inherently toxic in dose of 2 c.c., a smaller amount not being tested. On whipping, the blood defibrinated very slowly, after which it was centrifugated at 3000 revolutions; when tested at 18 minutes from the start of bleeding, it caused acute death in dose of 1 c.c., corresponding thus to tests Nos. 9 and 9a of Table 97.

Another like experiment was made with rabbit No. 3 of Table 69. After the first 2 tests which showed that its blood was inherently toxic in dose of 2 c.c., it was retested 3 days later, when its blood was found to be as toxic as before (Table 98, No. 3). It was then bled into a carotid pipet and the blood defibrinated with the rod; this took place very slowly, requiring about 8 minutes. The blood was then centrifugated at 8000 revolutions for 5 minutes and because of this high speed treatment it was not possible to make the first test until 47 minutes after the start of bleeding. It will be seen that at this point, which nearly corresponds to that of Nos. 10 and 10a of Table 97, 1 c.c. of the serum was practically without effect. A rapid series of tests with progressively increasing amounts of serum showed that very little of the original toxicity was left; a very delayed death ($1\frac{1}{2}$ hrs.) was obtained with 5 c.c., and even 7 c.c. required 47 minutes to produce a fatal result.

It is to be noted that while the heart blood was acutely fatal in dose of 2 c.c., the toxicity of the serum rapidly dropped off. It may be questioned whether this change was influenced by the prolonged defibrination, or by the use of the high speed centrifuge, or was merely due to the incident of time which, as seen from the table, ranged from 47 minutes to 2 hours and 19 minutes. The latter factor is probably the one which obtains.

A further experiment bearing upon the question at issue is presented in Table 99. For this series of tests a rabbit was bled into a carotid pipet, the bleeding time being 2'24"; of this whole blood, a portion of 3 c.c. was injected at once into guinea-pig No. 1, without any effect following. Unlike the bloods used in the 2 preceding tables, this was clearly non-toxic. The blood was then whipped for 5 minutes, and the resulting defibrinated blood was tested on No. 2 with fatal result, the toxicity being that developed incidental to the clotting

process. It was finally centrifugated at 3000 revolutions for 5 minutes, and the clear serum in dose of 3 c.c. was tested, 25 minutes after starting to bleed, with no effect. It will be seen from the table that while 3 c.c. of defibrinated blood (approximately 1.8 c.c. serum) sufficed to kill when injected 9 minutes from the start of bleeding, 6 c.c. of serum were necessary at 34 minutes. In other words, the defibrinated blood was more than 3 times as toxic as the cell- and suspenoid-free serum. The dose of 6 c.c. represents an average lethal dose and not a fatal one, as can be seen from tests Nos. 7 and 8, made after the serum was incubated at 37 C.

It would seem that, as in the case of anaphylatoxin production by agar, etc., the maximal toxicity is developed in the few seconds immediately preceding and following the clot formation. This rapid rise is

TABLE 99
COMPARATIVE TOXICITY OF A NORMAL RABBIT'S BLOOD AND SERUM, BEFORE AND AFTER
DEFIBRINATION

Guinea-Pig			Total Time*	Result
No.	Weight	Intravenous Injection (c.c.)		
1	200	3 whole blood	3' 6"	Nil
2	210	" defibr. "	3' 51"	5'
3	204	" serum	25'	Nil
4	178	6 "	34'	2' 51"
5	203	4 "	37'	Slight
6	190	5 "	42'	Very slight
7	200	6 " (15', 37 C.)	62'	Practically nil
8	205	6 " (30', ")	78'	8'

* The total time is from the start of bleeding into carotid pipet until end of injection.

followed by a fairly rapid fall, but the toxicity does not reach the low level of that of circulating plasma which can be considered as nil in the strictly normal rabbit, one whose blood is not inherently toxic. This drop is less complete if the serum remains in contact with the clot than if it is removed from such contact by centrifugation. Evidence substantiating this statement will be presented later on in the paper.

The cause of this rapid drop in toxicity was at first rather difficult to explain, since anaphylatoxic sera, in general, retain their poisonous quality even after long incubation. It is clearly associated with the removal of the suspended clot and cells. The highly toxic condition must be correlated with the sudden physical disturbance which occurs in the plasma just before, and during coagulation. The visible disturbance, that is, coagulation, is soon at an end, but the other dis-

turbance, that of toxicity, may persist for some time. The immediate cause of the coagulation of blood is the formation of an accelerator, commonly known as the fibrin ferment, which reacts with fibrinogen; likewise, the direct cause of the toxicity must be sought in a similar catalyzer which reacts with some very labile blood constituent. As a result, a certain amount of poison is produced, and this is in solution, since it remains in the serum after centrifugation; the greater part of the observed toxicity of whole, or of defibrinated blood is not due to such soluble poison, but rather to the accelerator which is injected with the blood into the animal.

TABLE 100

THE IN VIVO PRODUCTION OF ANAPHYLATOXIN IN RATS INJECTED WITH NORMAL RABBIT BLOOD (A), OR SERUM (B); TRANSFUSION TO GUINEA-PIGS

Series	Donor (rat)			Recipient (guinea-pig)		Total Time*	Transfer Time†	Result
	No.	Weight	Blood Injected (c.c.)	Weight	Rat Blood (c.c.)			
A	1	160	3 blood	210	2	1'15"	20"	Slight
	2	180	" "	185	"	1'12"	18"	"
	3	175	" "	195	"	2' 3"	28"	Near-kill
	4	200	" "	175	"	2'40"	31"	5'54"
	5	200	" "	170	"	3' 4"	34"	4'20"
	6	185	" "	175	"	4' 5"	25"	3'50"
	7	185	" "	210	1	4'15"	50"	Very severe
	8	125	" "	205	1	4'	20"	Slight
	9	210	1.5 "	180	2	2'55"	29"	Moderate
B	10	140	10 serum	183	2	8'45"	25"	Nil
	11	130	" "	204	2	4'20"	30"	Slight
	12	190	15 "	206	3	5'	35"	Very slight

* Total time is that from start of injection of rat until end of injection of guinea-pig. The time taken for the transfusion from the rabbit to the rat is not given, but it ranged from 2'25" to 3'20"; this is time from moment of starting to draw blood from the rabbit until end of injection of the rat, and was selected so as to allow pre-coagulation toxicity to develop in the rabbit blood.

† The transfer time means the interval from the entrance of the syringe into the heart of the rat until its withdrawal from the guinea-pig. It therefore represents the maximal time any of the rat blood was in the syringe, and it must be kept as short as possible.

The difference between the total and transfer time represents the reaction period during which anaphylatoxin may be made within the rat.

It has been pointed out in connection with Table 96 that the white rat is about as susceptible as the guinea-pig to the pre-coagulation toxicity. This clearly rules out of consideration such anaphylatoxin as may have been already developed in dose of blood injected, since the rat can withstand enormous doses of anaphylatoxin without any ill effect. If the toxicity of blood is associated with anaphylatoxin-production, it follows that the fatal results in rats must be due to the injection of an accelerator which then reacts with the large volume of

blood within the animal, and thus gives rise to the anaphylatoxin which causes death.

The effect of this accelerator upon the blood plasma is analogous to that of agar, peptone, kaolin, silicic acid, bacteria, trypanosomes, etc. These substances or cells, strictly speaking, are not toxic in themselves, but they induce a disturbance within the plasma or serum. This may be demonstrated in fresh blood by the method devised by Professor Novy and used first in the analysis of the action of several of the above agents. The results of the application of this method will be found in Table 100.

Inducing Power of Fresh, Undefibrinated Rabbit Blood.—Of all the sera that have been tested, that of the rat yields by far the highest toxicity when ordinary procedures for anaphylatoxin production are applied. On the other hand, the rat is very resistant to the action of anaphylatoxin, whether this is made from its own or from a heterologous serum. This high lability of rat serum allows one to demonstrate poison production, *in vivo*, by introducing the disturbing substance into the circulation of the rat, and then rapidly transfusing its blood into a guinea-pig.

For the tests given in Series A of Table 100, the rabbit blood was obtained by heart puncture, and then kept in the syringe for a varying length of time. This was 2 minutes for Nos. 1, 3, 4, 5, 9, and 3 minutes for Nos. 2, 6, 7, and 8. One rabbit was used for Nos. 1 and 2; another for Nos. 6, 7, 8; and a third for Nos. 3, 4, 5, and 9. After being thus kept in the syringe in order to reach the precoagulation stage, the blood was then injected into the femoral vein of large white rats, the dose being 3 c.c., except in the case of No. 9. Within a minute after such injection the rats usually showed very severe symptoms: respiratory trouble, dyspnea, and spasms. After the injection each rat was kept on the board and at a given period the heart was exposed and 2 c.c. of blood were withdrawn as rapidly as possible and at once injected into a guinea-pig. This transfer time was necessarily a trifle long on account of the marked drop in blood pressure of the rat; it was because of this difficulty that only 1 c.c. was transferred in the case of Nos. 7 and 8.

It is to be noted that, as in like transfusions after injection of agar (Table 65), or of peptone (Table 92), the result was slight in tests made with rat blood having a reaction time of less than a minute (Nos. 1 and 2), that is, where the blood was drawn before any symp-

toms had appeared. But as the reaction time, that in which the rabbit's blood remained in the rat's circulation, increased so did the severity of the effects in the guinea-pig; the effects were those of typical anaphylactic shock with typical findings in case of death. Careful examination of the guinea-pig heart and large vessels failed to disclose thrombi. It must be concluded, then, that the rabbit blood, when injected in the preclot stage into the circulation of the rat, renders the blood of the latter toxic for guinea-pigs. Such rabbit blood is not only poisonous, but also possesses an inducing power whereby the blood of the recipient is rendered toxic.

Inducing Power of Rabbit Serum.—Does this inducing activity persist after defibrination and centrifugation? It was very important to ascertain whether the inducing power, found to be present in the whole blood, persisted or was absent from the serum. To test this, experiments similar to the preceding were carried out, differing only in the substitution of rabbit serum for preclot blood. The results of such tests will be found under Series B, Table 100.

For test No. 10, a normal rabbit serum was employed, which, in dose of 3 c.c., was fatal to 2 of 3 guinea-pigs. Consequently, the rat which received 10 c.c. of this serum was given 3 guinea-pig lethal doses, without showing the slightest effect; the transfusion of 2 c.c. of blood from its heart likewise had no effect.

For tests Nos. 11 and 12, a rabbit serum was used which had been toxified by agar so that the lethal dose was 2 c.c. The two rats received 10 and 15 c.c. of this serum, respectively, which amounts correspond to 5 and $7\frac{1}{2}$ guinea-pig lethal doses of anaphylatoxin; they showed some respiratory disturbance and dyspnea. From the heart of each, 2 or 3 c.c. of blood were withdrawn and injected into guinea-pigs with practically no effect. As illustrating the effect of anaphylatoxin upon the coagulability of blood, it may be added that the blood remaining in the heart of these rats was perfectly fluid 6 minutes after the withdrawal of the test portion.

A comparison of the experiments in Series A and B shows a strikingly different picture. Large amounts of rabbit serum, representing 3 to $7\frac{1}{2}$ guinea-pig lethal doses, do not have the power to make the rat blood toxic, *in vivo*. The latter series serves, in a way, as control for the former; thus, it will be seen that with the same transfer time as used for Nos. 4 to 6, the transfusion of 2 or 3 c.c. of rat blood was without effect. For other control tests, showing the effect of the

transfusion of normal rat blood into guinea-pigs, reference is made to Table 66. Similar transfusion experiments will be found in Tables 18, 65, 92, and 134.

Apparent Variation in Toxicity of Normal Rabbit Serum.—The defibrination of rabbit blood by means of the glass rod failed to produce sera which were uniformly toxic for guinea-pigs. Although at rare intervals a serum that killed in 3 c.c. dose was obtained, usually the lethal dose of serum prepared by this method averaged 6 c.c. It was also noted that the toxicity of normal rabbit serum apparently was not constant, but seemed to fluctuate above and below a given fatal dose, just as in the case of anaphylatoxin prepared in diverse ways. This variation can easily be shown by testing a sample of serum at intervals of 15 minutes. Care must be taken, however, not to use too large a dose, since otherwise one would obtain an almost unbroken series of fatal results.

TABLE 101
APPARENT VARIATION IN TOXICITY OF NORMAL RABBIT SERUM, KEPT AT 38 C.; SUBACUTE DEATHS

Guinea-Pig		Serum		Result
Number	Weight	Intravenous Injection (c.c.)	Time at 38 C. (hr.)	
1	200	6	—	Slight
2	190	"	1/4	3' 5"
3	175	"	1/2	5'40"
4	205	"	3/4	Practically nil
5	199	"	1	11'
6	210	"	1 1/4	8'15"
7	207	"	1 1/2	Very slight

The first injection was made 28 minutes after start of bleeding, at which time the serum was placed at 38 C. The serum of one rabbit was used for all of the tests.

For the experiment given in Table 101, a rabbit was bled from the carotid; the blood was defibrinated with the glass rod for 5 minutes, then centrifugated at 8000 revolutions for 2 minutes; the resulting serum was pipetted off and a portion of 6 c.c. was tested at once, the balance being placed at 38 C. and tested at intervals shown in the table.

The subacute deaths in these tests indicate that the serum was but moderately toxic. It is noteworthy that the onset of symptoms was delayed for 2 to 4 minutes. The shock was typical of anaphylatoxin, as were also the autopsy findings. The absence of clot in the heart, the examination being made from 3 to 7 minutes after death, is deserving of special note. The results of this series of tests will be more evident by inspection of Chart XII.

The apparent variation in toxicity in this and similar experiments at first seemed to be too regular to be ascribed to a varying susceptibility of the test animals. In due course of time, however, it was realized that the guinea-pig was by no means as uniform a reagent as had been believed; hence the variation here, and in like tests, must be attributed to this factor and not to a change in the poison. Many experimenters in the past have drawn the erroneous conclusion that the toxicity of a serum had disappeared when one or two tests gave negative results. Marked variation in animals must be expected and abundant evidence of this will be presented.

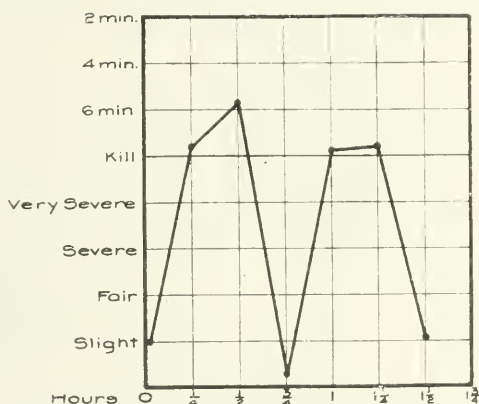


Chart 12. Apparent variation in toxicity of normal rabbit serum (Table 101).

Effects Produced by Normal Rabbit Serum.—While according to Doerr and Weinfurter,³¹ a lethal dose of 6 c.c. for 200 gm. guinea-pigs is high, Mita and Ito³² found 4 of 16 normal rabbits to yield sera which killed in dose of 2 c.c., moreover, they obtained 1 which was fatal in dose of 1 c.c. per 200 gm. Considerable effort was devoted to ascertaining the reason for this marked difference in results. Prepared by thorough rod defibrination, the sera gave results that were very disappointing. Occasionally, a serum could be obtained which was fatal in dose of 3 c.c. (Table 103), but this was the exception, rather than the rule. A year later, however, the problem was solved by the discovery that seemingly perfectly normal rabbits could possess a highly toxic, circulating blood. A striking example of this has been presented in Table 97, where it will be seen that even 1 c.c. of blood may be acutely fatal, and that the serum from such blood may also kill in this

dose. This, and like instances, show that the sera of normal rabbits may vary greatly in initial toxicity. It will be shown, however, that the method of defibrination exerts a marked effect upon the toxicity of a serum.

In the course of the early attempts to increase the toxicity of normal rabbit serum, 2 types of death were found. In one type, which was very acute, the animals succumbed in from 2 to 3 minutes, while in the second, or subacute type, death was delayed. In the latter, the appearance of the symptoms was usually retarded for from 2 to 4 minutes, and death occurred in from 8 minutes to 1 or 2 hours. In both types, the train of symptoms leading to the fatal result was the same. On the other hand, the findings at autopsy were different.

TABLE 102
APPARENT VARIATION IN TOXICITY OF NORMAL RABBIT SERUM, KEPT AT 38 C.; ACUTE DEATHS

Guinea-Pig		Serum		Result
Number	Weight	Intravenous Injection (c.c.)	Time at 38 C. (hr.)	
1	200	6	—	3'30"
2	175	"	1/4	2'35"
3	200	"	1/2	2'45"
4	182	"	3/4	2'55"
5	185	"	1	Slight
6	195	"	1 1/4	3'30"
7	215	"	1 1/2	Slight

The first injection was made 28 minutes after start of bleeding, at which time the serum was placed at 38 C. The serum of one rabbit was used for these tests.

In the rapid deaths, the lungs were found to be enormously distended and very light-colored. One rarely met with any congestion or even with ecchymoses. Further, the right heart almost invariably contained clots when an acutely fatal dose of 6 c.c. was injected. In the slower type the heart clot was absent; the lungs were also greatly distended, but instead of being white they were peppered with ecchymoses, which usually were present in greater number, the slower the death. Such lungs were also edematous. This condition reveals itself, just before death, by the exudation of large amounts of foamy fluid from the nose and mouth of the dying animal.

Jonesco-Mihaesti²⁴ described a similar difference when large and small doses of rabbit antish sheep red cell serum were injected into guinea-pigs. The first effect mentioned he obtained with large doses (5 to 6 c.c.), and the second with doses of 2 c.c. of the serum.

²⁴ Compt. rend. Soc. de biol., 1913, 74, p. 1414.

The tables which follow will indicate some of the variations met with when the same dose of different sera is used, and also the variations in picture that can be produced by employing variable doses of one serum. A series of rather slow deaths are given in Table 101; these results may well be compared with those in Table 102.

For the experiment given in Table 102, the serum of a rabbit was prepared in exactly the same way as for the tests in Table 101. The tests were likewise made under corresponding conditions, the first injection being given 28 minutes after the start of bleeding. It will be seen that the serum of this rabbit was more toxic than that used in the preceding series. This is evident from the larger number of deaths and the acuteness of the shocks. The onset of symptoms was immediate,

TABLE 103

TOXICITY OF A NORMAL RABBIT SERUM, KEPT AT ROOM TEMPERATURE: RELATION OF DOSE TO CLOTTING

Guinea-Pig		Serum		Result	Clot at Autopsy
Number	Weight	Intravenous Injection (c.c.)	Time at Room Temperature (hr.)		
1	200	6	—	3'	+
2	180	"	$\frac{1}{4}$	2'40"	++
3	177	"	$\frac{1}{2}$	2'45"	+++
4	171	"	$\frac{3}{4}$	2'	+
5	177	"	1	3'10"	++
1a	182	3	—	6'40"	0
2a	179	"	$\frac{1}{4}$	42'	0
3a	195	"	$\frac{1}{2}$	50'	0
4a	174	"	$\frac{3}{4}$	Very slight	—
5a	190	"	1	3'15"	++

The sign + indicates a very slight clot; ++, a fair one; and +++, much clot in heart at autopsy.

and the shock was typical of anaphylatoxin poisoning. A further and more striking instance of marked toxicity of a normal serum is given in Table 103.

Relation of Dose to Picture at Autopsy.—For the series of tests in Table 103, a rabbit was bled in the usual way, and, after rod defibrination, the blood was centrifugated at 8000 revolutions for 5 minutes. Tests Nos. 1 and 1a were made 50 minutes after start of bleeding; the other tests were made at quarter hour intervals, the serum being kept in the dark and at room temperature instead of at 38 C., as in the 2 preceding sets. The serum of a single rabbit was used for all of these tests.

The serum was toxic in dose of 3 c.c., and on keeping, it seemed to become less poisonous (Nos. 2a to 4a); test No. 5a, however, was more acutely fatal than the first, probably because the guinea-pig was more than usually susceptible, though of course it is possible that Nos. 2a to 4a were somewhat resistant. On the other hand, the 6 c.c. which represents about 2 average lethal doses, always produced acute death with more or less clot in the heart. The same serum, in 3 c.c. dose, produced no intracardiac thrombosis except in the case of No. 5a.

Other instances corresponding to test No. 5a were found where a dose of 3 c.c. of serum caused death and the heart showed a slight clot at autopsy, but such results were rather exceptional and imply either marked response on the part of the animal, or that more than one lethal dose was contained in the serum injected. It must not be inferred, however, that rapid postmortem clotting is due to much

TABLE 104

COMPOSITE TABLE SHOWING THE RELATION OF DOSE OF RABBIT SERUM TO TIME OF DEATH AND TO THE PRESENCE OR ABSENCE OF CLOT

No.	Serum (c.c.)	Time of Death	Clot	No.	Serum (c.c.)	Time of Death	Clot	No.	Serum (c.c.)	Time of Death	Clot
1	6	3'	+++	9	6	4' 5"	0	17	3	4'10"	⊕
2	"	2'30"	+++	10	"	4'55"	0	18	"	3'40"	0
3	"	1'50"	+++	11	"	8' 5"	0	19	"	4'30"	0
4	"	3'20"	+++	12	"	5'45"	0	20	"	16'20"	0
5	"	3'	+++	13	"	11'10"	0	21	"	3'25"	0
6	"	2' 5"	+++	14	"	8'25"	0	22	"	7'	0
7	"	2'10"	+++	15	"	7'55"	0	23	"	3'20"	0
8	"	3'45"	+	16	"	7'	0	24	"	6'40"	0

All injections were given intravenously. The sign ⊕ indicates a doubtful clot.

poison, but rather that it is due to an increased amount of accelerator or thrombin. It has been shown in Part V that many multiple doses of anaphylatoxin may be injected without causing clot formation; indeed, the reverse occurs. In view of this fact, it follows that early intracardiac clotting in deaths caused by serum is due to a residual inducing power, such as has been demonstrated in connection with whole blood. The injection of such blood into rats and guinea-pigs gives rise to an early clot.

The fact that large doses produce death with clot while smaller doses may kill without clot was noted by Doerr and Moldovan,²⁴ while working with rabbit immune serum. They did not attribute the death of such animals to thrombosis. In all of the guinea-pigs of this series, the lungs showed maximal distention; in the slow deaths the lungs

were peppered with hemorrhagic spots, while in the rapid cases they were pale.

By way of illustrating the constancy of the effects shown in Table 103, the results of a number of experiments are collated in Table 104. These were obtained with a number of different sera, tested on various occasions.

Antagonistic Action of Sodium Oxalate.—It has been shown that a rabbit serum which, in dose of 6 c.c., produces death with intracardiac clotting may be expected to kill, in a dose of 3 c.c., but without thrombosis of the heart and large vessels. It might be expected that by mixing such serum with sodium oxalate solution, a dose of 6 c.c. would cause death without the formation of clot. Experiments made with this object in view were successful as shown in Table 105.

TABLE 105
ANTAGONISTIC ACTION OF SODIUM OXALATE; CLOT PRODUCTION AND TOXICITY

Exper.	Guinea-Pig		Given Mixture of		Contact Time (min.)	Result	Clot
	No.	Weight	Serum (c.c.)	Oxalate (c.c.)			
A	1 C*	196	6	—	—	3'	++
	2	182	"	1	3	2'50"	0
	3	203	"	0.5	"	2'50"	++
B	4 C	198	"	—	—	3'35"	++
	5	182	"	0.75	3	1'45"	0
	6	205	"	1	6	3'	0
	7	201	"	1	9	Moderate	—
	8 C	184	"	—	—	3'15"	+++
	9 O	204	3	—	—	3'15"	0
	10 O	194	6 salt	1	—	Nil	—
C	11 C	180	6	—	—	4'	+++
	12	189	"	1	3	3'50"	+++
	13	200	"	1	8	3'	0

* The letter C after number implies that the test is a control.

In the experiments given in this table, 3 different normal rabbit sera were employed, and each killed in 6 c.c. dose with intracardiac clot formation. This amount of serum was mixed with varying quantities of a 1% sodium oxalate solution (in 0.85% salt solution) and allowed to stand at room temperature, the duration of contact being indicated in the table; the mixture then was injected intravenously. The fine precipitate of calcium oxalate that formed at once upon the addition of the oxalate is not a factor in the death of the animals, since its removal by centrifugation does not in any way affect the result. When centrifugation was employed, it necessarily lengthened the contact time

for the mixture (tests Nos. 6, 7, and 13). It may be added that control tests with 1 or 2 c.c. of oxalate diluted with salt solution had no effect. The autopsies were made as always, 3 minutes after death.

The serum employed for Exper. A, as shown by control (No. 1), killed in dose of 6 c.c. with clot formation. This serum was also fatal in 3 c.c. dose, but in that case no clot resulted; 1 c.c. of oxalate prevented clot formation, while 0.5 c.c. did not do so.

In Exper. B, where a different serum was used, controls Nos. 4 and 8 show that in 6 c.c. dose it killed with presence of clot, while 3 c.c., though fatal, caused no clot (No. 9). Here again it will be seen that 0.75 and 1 c.c. of the oxalate, while not preventing death did do away with clot formation (Nos. 5 and 6). Test No. 7 produced only a moderate shock, the contact time in this case being 9 minutes. Subsequent control tests (Nos. 8 and 9) showed that the serum was as active as in the beginning. The harmlessness of an oxalate salt mixture is shown in control test No. 10.

In Exper. C, which was made several days later, the serum killed in dose of 2 c.c., without clot, while in dose of 6 c.c. it was fatal, with clot production (No. 11). This amount of serum, therefore, represents 3 lethal doses; when mixed with 1 c.c. of oxalate, a contact of 3 minutes did not prevent clot, whereas one of 8 minutes did.

The above results indicate that an oxalate-serum mixture, with a contact of 3 minutes, may kill without clot, provided the amount of serum does not represent more than 2 lethal doses. If the toxicity is greater than this, a longer contact appears to be necessary. And, further, with a sufficiently long contact the serum may become non-fatal. It would seem as if the oxalate exerted an antagonistic action, first, as to the clotting factor, and second, on the toxicity.

In this connection it may be stated that a fresh oxalate plasma which was nontoxic in dose of 3 c.c., became acutely fatal in the same dose when tested within a few minutes after recalcification. In this case the oxalate inhibited the toxicity as well as the coagulation. A further illustration of this action of oxalate is seen when rabbit blood, drawn from the heart and kept in the syringe for 3 to 3¼ minutes, is added to a sodium oxalate solution. As shown in Table 96, such whole blood, in a dose of 3 c.c., will cause either a severe shock or an acute death; the oxalate mixture, however, does not clot and is not toxic. Blood thus oxalated, when injected at 5 and at 11 minutes after start of bleeding, was without effect (3.3 c.c.).

Effect on Toxicity of Spontaneous Clotting: Minimal Contact with Clot.—Most variable results, as to toxicity, were obtained when the blood was defibrinated by means of the glass rod. As a rule, the fatal dose of the serum thus obtained rarely goes below 6 c.c. On the other hand, as mentioned, Mita and Ito obtained several rabbit sera which were fatal in dose of 2 c.c., and one which killed in 1 c.c. The latter was fatal in 7 tests made from $\frac{1}{2}$ to 5 hours after bleeding. They make no mention of the way in which they clotted the blood for their tests. They do state that the rabbits were bled from the carotid, and that the blood was at once centrifuged and tested immediately. The serum was then kept at room temperature in the dark, and retested at intervals. We were led to infer, therefore, that the blood was allowed to clot spontaneously in the centrifuge tube.

Our first attempt to influence toxicity, by varying the time and kind of clot contact, consisted in trying to duplicate as closely as possible the time relations in Mita and Ito's experiments.

In one experiment the rabbit was bled white in 3 minutes and the blood was transferred at once to 3 centrifuge tubes. Within $8\frac{1}{2}$ minutes after start of bleeding the centrifuge had reached 8000 revolutions, and this speed was maintained for 5 minutes. Under these conditions the clot was removed as fast as formed. Indeed, in one instance the cell-free layer showed a clot at the end of centrifugation. In 22 minutes after starting to bleed, the first injection was made. The serum was then kept in the room and retested as indicated.

The results of this experiment are given in Table 106, A. The effect here was no better than with rod defibrination. Since the toxicity of a serum does not increase when kept at room temperature, the experiment was discontinued.

Other attempts were made with this method of rapid removal of the spontaneous clot, but they were productive of no better result. While serum obtained in this way will not kill in dose of 3 c.c., it will do so regularly with 6 c.c. It was evident from these tests that the cause of increased toxicity must lie in some other direction. An attempt was made to reach a greater degree of toxicity by allowing the blood to remain undisturbed in contact with the fibrin of the spontaneous clot. It seemed possible that such contact with the extensive fibrin surface might influence the result.

Contact for Thirty Minutes with Spontaneously Formed Clot.—The results of an experiment of this kind are given in Table 106, B. The

rabbit was bled as usual into a carotid pipet, the blood was transferred at once to 3 centrifuge tubes, and allowed to clot spontaneously. It remained thus at room temperature for 30 minutes, when it was centrifuged at 8000 revolutions for 15 minutes. The first test of the clear serum was made 72 minutes after the start of bleeding.

The serum thus prepared was fatal in dose of 6 c.c., but 3 c.c. had very little effect. Clearly, a long contact was no better than a minimal one. Possibly a long contact with the fibrin caused a reversion change;

TABLE 106
EFFECT OF SPONTANEOUS CLOTTING ON TOXICITY OF RABBIT SERUM; MINIMAL CONTACT WITH CLOT (A); CONTACT FOR 30 MINUTES (B); CONTACT FOR 20 MINUTES (C)

Exper.	Guinea-Pig		Rabbit Serum		Result
	Number	Weight	Intravenous Injection (c.c.)	Time at Room Temperature (hr.)	
A	1	196	3	—	Moderate
	2	185	6	—	4/30"
	3	208	3	1/4	Very slight
	4	195	6	1/4	3/15"
B	5	180	6	—	2/35"
	6	194	3	—	Moderate
	7	192	6	1/4	3/10"
	8	203	3	1/4	Very slight
	9	191	6	1/2	Severe
	10	201	6	3/4	4/35"
	11	207	6	1	3/40"
	12	192	6	2 1/2	2/40"
	13	200	3	"	Slight
C	14	198	3	—	3/15"
	15	198	2	—	Very slight
	16	175	3	1/4	3/10"
	17	196	3	1/2	Very slight
	18	200	3	3/4	2/50"

In Exper. A, the first 2 injections were made 22 minutes after start of bleeding; in Exper. B, this time was 72 minutes; and in Exper. C, it was 51 minutes. The other tests were made at subsequent intervals, as indicated.

to test this, the experiment was repeated with shorter contact time, about midway between those given in Expers. A and B; hence, a contact time of 20 minutes was taken for the next trial.

Contact for Twenty Minutes with Spontaneously Formed Clot.—An experiment of this kind will be found in Table 106, C. The blood of a rabbit was blown at once into centrifuge tubes and these were left at room temperature for 20 minutes, after which they were centrifuged at 8000 revolutions for 15 minutes. The clear serum was pipetted off

and tested at once, the balance being kept at room temperature and tested at intervals, as shown in the table.

The increase in toxicity was realized, since, as seen from the table, out of 4 injections of 3 c.c. of such serum, 3 resulted in acute and typical death. This serum was used 4 hours later for Exper. A, Table 105, and a similarly prepared serum was employed for Exper. B in the same table; that used in Exper. C, likewise made in this way, killed in dose of 2 c.c. a 181 gm. guinea-pig in 62 minutes, the findings being typical. This experiment was repeated at other times with like result. In only one instance did such serum fail to kill in dose of 3 c.c., the serum in that case being very lipoidal.

To sum up these experiments upon the toxicity of serum obtained from spontaneously clotted blood, we may say that both short (8 minutes) and long contact (30 minutes) fail to produce a very toxic serum. On the other hand, a contact of 20 minutes with the fibrin network yields a serum which quite consistently kills in dose of 3 c.c., and once was fatal in 2 c.c. dose, the highest toxicity which we were then able to secure. At the time this work was done it was believed that the rabbit blood was fairly uniform as to its capacity for poison production, but a year later it was found that such was not the case, and that rabbits could possess an inherently toxic blood, one which, on rod defibrination, was fatal in dose of 1 c.c. (Table 97).

Effect on Toxicity of Bead Defibrination at Room Temperature.—Moldovan,¹¹ testing the toxicity of freshly defibrinated rabbit blood for homologous animals, claimed that a vigorous shaking with glass beads for 5 minutes was necessary to make blood toxic. He stated that the failure of Blaizot³⁵ to detect the toxicity in normal rabbit blood was probably due to the fact that he did not shake it vigorously enough with beads. Studzinski,³³ in testing the effect of fresh homologous blood on dogs, came to the conclusion that energetic shaking with beads was necessary to liberate the toxic substance, which he believed to come from the erythrocytes.

We have tested the effect of bead defibrination upon the toxicity of normal rabbit serum for guinea-pigs, and although we consider the use of beads an important factor, we do not think that very vigorous or very prolonged shaking is of prime importance.

For the experiment given in Table 107, a rabbit was bled white from the carotid, and the blood was blown at once into an Erlenmeyer

³⁵ Compt. rend. Soc. de biol., 1910, 68, p. 1124.

flask of about 500 c.c. capacity, the bottom of which was covered to a depth of from 5 to 6 mm. with glass beads. The flask was then shaken vigorously at room temperature for 2 minutes, at the end of which time the coagulation appeared to be complete. A portion of the blood was then transferred to a centrifuge tube and whirled at 3000 revolutions for 12 minutes; tests Nos. 1, 2, and 3 were made with this serum. The bulk of the blood was left in contact with the beads and clot at room temperature for two hours, when it was centrifuged and the serum used for Nos. 4 and 5.

The serum which remained in contact with the beads, clot and cells (No. 4) was apparently more toxic than that which was not. The failure to kill in Nos. 1 and 3 must be ascribed to the individual resistance of the test animals. At autopsy, slight clots were found in the hearts of both fatal shocks, a result rarely encountered with like dose

TABLE 107
EFFECT OF BEAD DEFIBRINATION, AT ROOM TEMPERATURE, ON THE TOXICITY OF RABBIT SERUM

Guinea-Pig		Serum		Result
Number	Weight	Intravenous Injection (c.c.)	Time at Room Temperature (hr.)	
1	182	3	—	Fair
2	184	"	1 $\frac{1}{4}$	7'45"
3	181	"	1 $\frac{3}{4}$	Slight
4	202	"	2	2'00"
5	180	2	2	Slight

Test No. 1 was made 23 minutes after the start of bleeding.

of serum prepared by rod defibrination. This would indicate that the grinding action of the beads may liberate more of the accelerator than is the case when the rod is used.

Effect on Toxicity of Bead Defibrination at 40 C.— Since the production of anaphylatoxin occurs more readily at the body temperature, or slightly higher, we tested the effect of coagulation at 40 C. Since the production of a toxic serum by the addition of agar occurs with facility at this temperature, it seemed that the same might be the case if fibrin made its appearance in the blood under similar conditions.

The results of 2 experiments of this kind will be found in Table 108. For the first of these, the rabbit blood was transferred at once from the carotid pipet to a flask with beads, which had been warmed for 15 minutes in a water-bath at 40 C. The flask was then shaken in the water-bath for 2 minutes. The larger part of the blood thus defibri-

nated was then centrifuged at 8000 revolution for 5 minutes, while a small bulk was whirled at 3000, in order to secure an earlier test than is possible with the high speed machine. This second portion, designated as A, was therefore tested first, while that centrifuged at 8000 is marked B.

It would appear from the results, as given in Table 108, that the very rapid bead defibrination at 40 C. yields a distinctly nontoxic serum, at least as to the dose employed. A similar unfavorable result was obtained in the second experiment, where the blood was added to the bead flask, previously warmed at 40 C. for 10 minutes; but instead of agitating the flask in the water-bath it was shaken in the room. The

TABLE 108
EFFECT OF BEAD DEFIBRINATION, AT 40 C., ON THE TOXICITY OF RABBIT SERUM

Exper.	Portion.	Guinea-Pig		Serum		Result
		No.	Weight	Intravenous Injection (c.c.)	Time at Room Temperature (hr.)	
I	A	1	180	3	—	* Very slight
	B	2	189	"	1/4	" "
		3	201	"	1/2	Nil
		4	185	"	3/4	Slight
		5	205	"	1	Very slight
II	A	6	180	"	—	3'30"
		7	210	"	1/4	Severe
	B	8	205	"	1/4	Slight
		9	178	"	1/2	"
		10	182	"	3/4	Severe
		11	200	"	1 1/4	Very slight
		12	189	"	1 1/2	Nil

The time from the start of bleeding to the injection of No. 1 was 16 minutes, while that of No. 6 was 17 minutes.

defibrination was, therefore less rapid than in Exper. 1, and the portion of the blood centrifuged at low speed caused an acutely fatal shock (slight clot in heart); on the other hand, portion B, which was centrifuged at 8000 revolutions for 5 minutes, had but a slight effect, except in No. 10, where a severe shock resulted.

In the preceding experiments, the blood was centrifugated in toto, and the serum freed from contact with the clot as soon as possible. A different result would have been obtained if it had remained in contact with the beads and clot, and at intervals small portions had been removed, centrifuged and tested. This, however, does not improve the result as will be seen on reference to Exper. A, Table 112.

From the standpoint of securing high toxicity, this method must be considered a complete failure. The poor results are not entirely due to the individuality of the rabbits employed, but depend rather on the temperature at which coagulation occurs (Table 112). It would be of interest to know whether, under these conditions, the serum would prove equally inert in 6 c.c. dose. To have a serviceable method which would give with some regularity a nontoxic serum would be a distinct advantage. It will be shown later that blood defibrinated by this method may be quite nontoxic, since 10 and even 12 c.c. may be without action on some guinea-pigs.

Effect on Toxicity of Chilled Bead Defibrination.—The failure to obtain the desired increase in toxicity by defibrination with beads at 40 C., and the fairly good results secured by spontaneous clotting suggested trials with chilled beads. It is well known that the chilling of freshly drawn blood retards to a considerable extent the progress of coagulation. It was possible that the state of fibrinogen, as it changes from its dispersed form to the coarser aggregates that make up the fibrin, was as important to the initiation of the poison-formation as that of the agar, or other suspenoid used in anaphylatoxin production. If such were the case, by retarding the coagulation and thus keeping the aggregating fibrin or threads in the finest possible state, results as good, if not better than those obtained by spontaneous clotting with sera, might be expected. Hence, the attempt was made to bring on the clot slowly by exposure to cold. It will be seen from the following experiments that the slow coagulation of rabbit blood is an important factor in toxification.

For the experiment shown in Table 109, a flask containing the usual layer of beads was iced for 30 minutes. A rabbit was then bled into a carotid pipet (3 minutes) and the blood was at once blown into the iced flask, which was then allowed to stand at room temperature for 5 minutes. At the end of this time, a beginning coagulation showed itself in the form of a slight gel. This was easily broken up by gentle swinging for 2 minutes, the clot collecting upon the beads. The flask, with the blood in contact with the fibrin-coated beads, was then kept at room temperature, and, at intervals, portions of 12 c.c. were drawn off, centrifuged at 3000 revolutions, and the serum thus obtained was tested for its toxicity. Seven portions were thus centrifuged; these are indicated in the table by the corresponding numbers. The results of this experiment should be compared with those given in Table 113.

This method gave the best results yet obtained. That it was not a matter of chance, due to an individual peculiarity of the rabbit used, will be shown in comparative tests to be presented later. An inspection of the table shows that this serum killed rapidly in dose of 3 c.c., while that of 2 c.c. was acutely fatal once, and caused very severe shocks in 3 of 4 other tests. The variable resistance of guinea-pigs accounts for the slight effects seen in Nos. 5 and 6. In 2 experiments, similar to that of Table 109, the serum tested 17 minutes after start of bleeding proved fatal in dose of 2 c.c. In one of these the toxicity, tho sub-acute, persisted through 4 consecutive tests made during the first hour. In this experiment the shaking with the beads was very gentle, yet contrary to the idea of Moldovan,¹¹ the toxicity was extremely high.

TABLE 109
EFFECT OF CHILLED BEAD DEFIBRINATION ON THE TOXICITY OF RABBIT SERUM

Guinea-Pig		Serum		Result
Number	Weight	Intravenous Injection (c.c.)	Time* at Room Temperature	
1	185	3	18 min.	2'15"
1a	179	2	22 "	Severe
2	185	3	23 "	2' 5"
2a	178	2	21 "	3'45"
3	182	2	40 "	Severe
4	178	2	1 hr. 9 min.	Near-kill
4a	182	1.5	1 " 11 "	Slight
5	184	2	2 " 30 "	Very slight
5a	180	3	2 " 33 "	Severe
6	190	3	3 " 9 "	Very slight
7	176	3	4 " 36 "	4'

* The time given for each test is that from the start of bleeding.

The pooled sera from the several portions (Nos. 1 to 4) used in the above experiment were subsequently treated with agar by the sol-gel method in order to see if such initially toxic serum could be toxified still more. The agar-serum mixture was placed at 37 C., and portions were removed, centrifugated and tested consecutively at $\frac{1}{4}$, $\frac{1}{2}$ and 1 hr., respectively. The first portion, in dose of 2 c.c., gave a very severe shock; the second and third portions in like dose killed in 4'50" and 2'35", respectively, while a test of 1 c.c. after incubation for 1 hour gave a severe shock. A similar test experiment made with the serum used in Exper. C, Table 105, also gave a 2 c.c. lethal dose after

incubation for 45 minutes at 37 C. In a third attempt, the serum had been prepared by spontaneous clotting (20 minute contact) and was toxic in 3 c.c. dose; treated by the sol-gel method (Mixture No. 6), and incubated at 37 C. for 15 minutes, it caused typical shock and death in 5 minutes in dose of 2 c.c. It appears, therefore, that the high initial toxicity of rabbit serum can be slightly increased by treatment with agar.

Comparative Toxicity: Rod and Bead Defibrination.—On account of the possible and actual variation in the quality of the blood in different rabbits, a strict comparison of the two methods of defibrination cannot be made with sera from bloods of different origin. It is essen-

TABLE 110
COMPARATIVE TOXICITY OF SERUM FROM ONE RABBIT: PORTION A, DEFIBRINATED WITH ROD;
PORTION B, WITH CHILLED BEADS. CHILLED ROD SERUM (C)

Exper.	Guinea-Pig		Serum		Result
	Number	Weight	Intravenous Injection (c.c.)	Time* at Room Temperature	
A	1	201	3	24 min.	Very slight
	2	175	"	44 "	Nil
	3	194	"	1 hr.	Slight
	4	176	"	1 hr. 24 min.	"
B	5	199	"	27 min.	4' 5"
	6	195	"	41 "	3'30"
	6a	180	2	47 "	67 min.
	7	180	3	1 hr. 4 min.	3'20"
	8	200	"	1 " 27 "	Very severe
C	9	187	3	24 min.	Very slight
	10	210	"	38 "	" "
	11	180	"	52 "	Nil
	12	181	"	1 hr. 10 min.	Very slight

* The time given for each test is that from start of bleeding.

tial that the blood for both methods should be obtained from the same animal and at the same time; moreover, the time relations should be as close as possible. With these conditions in mind, the following experiment was planned and carried out.

As a preliminary control for Expers. A and B of Table 110, 10 c.c. of blood were obtained from a rabbit by direct heart puncture, and injected into a guinea-pig of 210 gm. within 30 seconds after starting to draw the blood (Table 96, No. 8). The effect was but slight, since only a few mild spasms resulted. The dose given represented 6 c.c.

of plasma; consequently, it was to be expected that the serum would have a low normal toxicity.

The same rabbit was then bled white from the carotid and the blood was at once divided into 2 portions: one of which (A) was immediately defibrinated for 5 minutes with the glass rod, while the other portion (B) was blown into a flask with beads previously chilled by being kept outside the window at -7°C . The flask was then allowed to stand at room temperature for 6 minutes until a soft gel began to form, when it was shaken very gently for 2 minutes.

Both portions, A and B, were then kept at room temperature, and at intervals a sample was removed from each and centrifuged at 3000 for 6 minutes. The 2 sera thus obtained were then injected, the difference in time being, at most, 3 minutes. The results of these parallel tests are given in Table 110, and are also shown in Chart XIII.

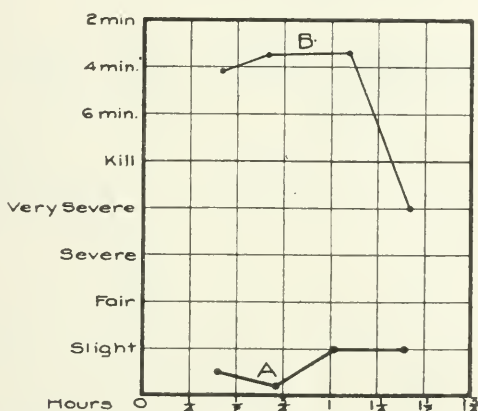


Chart 13. Comparative toxicity of normal rabbit serum: A, rod defibrination; B, chilled beads (Table 110).

This experiment constitutes a perfect comparison of the 2 methods of defibrination. The serum obtained by rod defibrination (A), in four tests, is shown to be practically innocuous in the dose employed. On the other hand, the serum prepared by chilled bead defibrination, from the same blood and under parallel conditions, was very toxic, since in a dose of 3 c.c. it caused 3 acute deaths; even 2 c.c. proved fatal. The conclusion, therefore, to be drawn is, first, that the toxicity of a serum depends on the mode of defibrination; second, that the method of rod defibrination yields a less toxic serum than that of chilled beads.

By way of summary it may be stated at this point that of 14 rod sera, one failed to kill in dose of 6 c.c., in 5 consecutive tests, made at intervals of 15 minutes; 3 of these sera caused subacute deaths, the animals dying as late as 70 minutes. It is evident, therefore, that the rod serum is the least toxic, while that obtained with chilled beads is the most active. The serum from a spontaneous clot, with a contact of 20 minutes, appears to occupy an intermediate position. The occasional poor success with the best method is indicative of individual variation in the rabbit blood.

Effect on Toxicity of Chilled Rod Defibrination.—Inasmuch as it was possible that the poor result with rod defibrination obtained in the preceding experiment was due to the warmer glass surfaces, it appeared desirable to make a control test in which the receiver with its rod was likewise chilled before use.

For this purpose a rabbit was bled through a cannula inserted into the carotid directly into an iced receiver. The latter consisted of a test-tube on foot, plugged with cotton and provided with a glass rod for the purpose of defibrination; before receiving the blood it was thoroughly chilled by being packed in cracked ice. After being filled with blood it was kept in the room for 6 minutes, until beginning coagulation; the blood was then whipped for 4 minutes, till coagulation was complete. The blood thus defibrinated was kept at room temperature, and at intervals portions were removed, centrifuged and tested. It will be noted that contact of the serum with the clot and corpuscles was thus maintained the same as in the previous experiments.

The results are given in Table 110, Exper. C. They are essentially the same as those of Exper. A. Although coagulation was retarded by the chilling of the receptacle, the toxicity was not increased. The explanation for this difference in the action of chilled beads and chilled rod must lie in the physical conditions presented in the 2 methods. In rod defibrination the clot is rolled up into a solid mass, whereas, in the former method, the fibrin is deposited on the beads; this enormous surface of fibrin and glass may be looked upon as a determining factor. It is conceivable that the large soft mass of fibrin which is gathered up by the rod, in being whirled through the blood, removes by adhesion or adsorption the suspended accelerator, and thereby prevents marked toxification of the serum; the removal of such a catalyzing substance would be less complete in the bead method where clotting is retarded and the agitation is of a gentle nature.

Effect on Toxicity of Rapid Clotting with Chilled Beads.—When the blood is caused to clot rapidly in an iced flask containing beads, the toxicity does not reach as high a level as when the clotting is retarded. This rapid clotting can be brought about by bleeding the rabbit slowly into a carotid pipet. In the most successful experiments with chilled beads, the blood was received into pipets which had not been chilled. The operation of bleeding usually lasted from 2 to 3 minutes, and the blood as soon as drawn was blown into the chilled bead flask. If, however, the bleeding operation took from 4 to 5 minutes, coagulation was already beginning to take place when the blood was introduced into the flask. The low temperature of the flask was not sufficient to check the clotting when this had once begun. The serum obtained under these conditions is no more toxic than that obtained by rod defibrination. An experiment of this kind is reproduced in Table 111.

TABLE 111

EFFECT ON TOXICITY OF RAPID CLOTTING WITH CHILLED BEADS: SERUM PORTION A WAS KEPT AT ROOM TEMPERATURE; PORTION B, AT 37 C.

Exper.	Guinea-Pig		Serum		Result
	Number	Weight	Intravenous Injection (c.c.)	Interval (hr.)	
A	1	195	3	—	Fair shock
	2	185	"	$\frac{1}{4}$	Very slight
	3	198	"	$\frac{1}{2}$	Fair
	4	189	"	$\frac{3}{4}$	Moderate
	5	182	"	1	Fair
B	6	173	"	$\frac{1}{4}$	Slight
	7	180	"	$\frac{1}{2}$	Severe
	8	201	"	$\frac{3}{4}$	Slight
	9	178	"	1	Very slight

No. 1 was injected 35 minutes after start of bleed; the others were tested subsequently at intervals given.

For the tests in the table mentioned, a rabbit was bled, 5 minutes elapsing from the start to complete exsanguination. The blood was blown at once into a flask containing beads, which had been thoroughly cooled by being packed in cracked ice. The surface of the blood set into a gel almost immediately after being transferred. The flask was shaken gently for 1 minute, when defibrination was complete. The blood was then transferred to centrifuge tubes and whirled at 8000 revolutions for 5 minutes. The serum thus obtained was tested at once (No. 1), while the remainder was divided into 2 portions: Por-

tion A was kept at room temperature, while portion B was placed at 37 C. The subsequent tests were made at intervals of 15 minutes.

In this experiment, the serum was not in contact with the clot and beads for more than 7 minutes at the time when centrifugation began. This condition may account for the rather feeble toxicity, since fractional centrifugation, as in Tables 109, 110, and 113, has been shown to give a highly toxic serum. However, reference to Table 112 will show that blood which is rapidly clotted with beads at 40 C. does not become toxified, notwithstanding fractional centrifugation. It would seem, therefore, that the large mass of clot formed under the condition of the above experiment removed the activating substance from the serum in much the same way as in rod defibrination.

TABLE 112

COMPARATIVE TOXICITY OF SERUM FROM ONE RABBIT: PORTION A, DEFIBRINATED WITH BEADS AT 40 C.; PORTION B, WITH BEADS AT 0 C.

Exper.	Guinea-Pig		Serum		Result
	Number	Weight	Intravenous Injection (c.c.)	Interval (hr.)	
A	1	174	3	—	Very slight
	2	201	"	$\frac{1}{4}$	" "
	3	207	"	$\frac{1}{2}$	Nil
	4	190	"	$\frac{3}{4}$	Very slight
	5	210	"	1	Slight
	6	207	"	$1\frac{1}{4}$	
B	7	175	"	—	5'25"
	8	194	"	$\frac{1}{4}$	4'40"
	9	202	"	$\frac{1}{2}$	Moderate
	10	205	"	$\frac{3}{4}$	Very severe
	11	201	"	1	5'20"
	12	203	"	$1\frac{1}{4}$	Very slight

The interval from the start of bleeding to the injection of Nos. 1 and 7 was 33 and 30 minutes, respectively; the other tests followed at the stated time.

Comparative Toxicity: Bead Defibrination at 40 and at 0 C.—The experiment summarized in Table 108 indicated that blood which was transferred at once after drawing to a flask with beads that had been kept at 40 C., and then defibrinated at this temperature, did not become highly toxic. To avoid once more the objection that we might be dealing here with an individuality as regards the blood source, the experiment given in Table 112 was devised.

For this experiment a rabbit was bled into a carotid pipet, and the blood was transferred at once to 2 flasks containing beads: One of these (A) had been immersed in water at 40 C.; this flask was at once

gently shaken for 2 minutes when defibrination was complete. The other flask (B) had been packed in crackle ice for some time before addition of blood; hence, coagulation was retarded; the clotting began after the blood had stood in this flask at room temperature for 8 minutes. The flask was then shaken for 2 minutes in the same way as flask A. The 2 flasks were then kept in the room, and at intervals of 15 minutes portions of 6 c.c. were removed from each and centrifugated side by side at 3000 revolutions for 5 minutes, after which each serum was tested, the dose being 3 c.c. Of each blood, 6 portions were thus centrifugated and tested.

The results of this experiment are shown in Table 112 and in Chart XIV. They present a striking demonstration of the effect upon toxicity of slow and rapid coagulation of one and the same rabbit

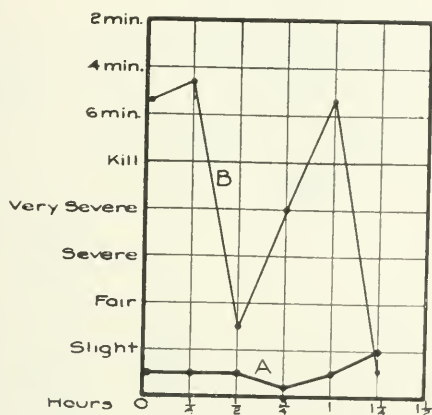


Chart 14. Comparative toxicity of normal rabbit serum: A, beads at 40 C.; B, at 0 C. (Table 112).

blood. The difference here is just as marked as in Expts. A and B of Table 110. The rapid and complete removal of clot, either by rod or by warmed beads, yields a serum of less toxicity than that when chilled beads are employed.

Toxicity of Serum Kept in Contact with Clot.—In a number of experiments recently considered (Tables 109, 110, and 112) the blood was kept in contact with the clot, and portions were centrifuged as needed. The toxicity of a serum thus maintained in contact with its clot is distinctly higher and persists longer than seems to be the case with serum which has been centrifuged out of a blood, *en masse*, and then kept at room temperature. Continued contact of the serum with

either fibrin or a suspended catalyzer at a suitable temperature is needed to develop high toxicity, the condition being exactly similar to that of anaphylatoxin-production by agar or other suspended matter.

The experiment given in Table 113 is intended to show the persistence of toxicity in a serum kept in contact with its clot at room temperature. For this series of tests a rabbit was bled into a carotid pipet (2 minutes) and the blood was transferred at once to a bead flask which had been iced for half an hour. The surface gel appeared in $5\frac{1}{2}$ minutes, whereupon the flask was swung for 1 minute. The blood thus defibrinated was now allowed to stand at room temperature; at intervals of 15 minutes, a portion of 6 c.c. was removed and centrifuged at 3000 for 5 minutes; the resulting serum was then tested in 3 c.c. dose. Ten such portions were tested.

TABLE 113
PERSISTENCE OF TOXICITY IN SERUM KEPT IN CONTACT WITH CLOT; CHILLED BEAD
DEFIBRINATION

Guinea-Pig		Serum		Result
Number	Weight	Intravenous Injection (c.c.)	Interval (hr.)	
1	185	3	—	Very severe.
2	180	"	$\frac{1}{4}$	3'25"
3	195	"	$\frac{1}{2}$	4'25"
4	179	"	$\frac{3}{4}$	7'
5	184	"	1	3'20"
6	202	"	$1\frac{1}{4}$	Very severe
7	178	"	$1\frac{1}{2}$	5'15"
8	180	"	$1\frac{3}{4}$	7' 5"
9	187	"	2	4'20"
10	180	"	$2\frac{1}{4}$	4 hr.

The interval from the start of bleeding to the injection of No. 1 was 20 minutes; the other tests were made at subsequent intervals, as given.

The results, as presented in Table 113, show that under these conditions the toxicity remains constant for at least $2\frac{1}{2}$ hours after start of bleeding. The failure to kill acutely in 3 of 10 tests merely indicates greater resistance on the part of the test animal. In view of this high average toxicity, it is probable that the serum would have killed some guinea-pigs in a dose of 2 c.c. The centrifugation of the first portion was commenced 13 minutes after the start of bleeding, which gives a contact time of only about 8 minutes; it is likely that even at this time the full toxicity was developed, though test No. 1 failed to kill.

Toxicity of Serum Freed from Contact with Clot.—It has been pointed out in connection with Table 111 that the early removal of the

serum from contact with clot and corpuscles may explain the feeble toxicity observed in that experiment. It is also possible that the absence of toxicity in such serum was due to the quality of the blood which happened to be used in that experiment. It has been shown in Part III that the reactivity of rabbit serum is subject to considerable variation, and it is not unlikely that this factor holds true for the poison production incidental to blood coagulation.

As a further illustration of the apparent effect of centrifugation on toxicity, the experiment shown in Table 114 is given. It is to be noted that the conditions, except as to centrifugation, are essentially the same as those for the experiment presented in Table 113; the 2 experiments were made on consecutive days, and necessarily the bloods were of different origin.

TABLE 114

APPARENT DECREASE IN TOXICITY OF SERUM AFTER REMOVAL FROM CONTACT WITH CLOT;
CHILLED BEAD DEFIBRINATION

Guinea-Pig		Serum		Result
Number	Weight	Intravenous Injection (c.c.)	Interval (hr.)	
1	192	3	—	3'35"
2	202	"	1 $\frac{1}{4}$	Moderate
3	200	"	1 $\frac{1}{2}$	Severe
4	185	"	3 $\frac{1}{4}$	"
5	205	"	1	Fair
6	201	"	1 $\frac{1}{4}$	Very slight
7	200	"	1 $\frac{1}{2}$	Slight
8	195	"	1 $\frac{3}{4}$	Very severe
9	187	"	2	Slight
10	175	"	2 $\frac{1}{4}$	Moderate
11	205	"	2 $\frac{1}{2}$	Nil
12	200	"	2 $\frac{3}{4}$	Moderate
13	186	"	3	Slight

The interval from the start of bleeding to the injection of No. 1 was 52 minutes; the other tests followed as stated.

For this experiment a rabbit was bled into a carotid pipet (2 minutes) and the blood was blown at once into a bead flask previously chilled in ice. As soon as the clot began to appear on the surface, which was in 5 minutes, the flask was swung for 1 minute. Upon standing for a minute an additional clot appeared, whereupon it was shaken for another half minute. The blood was then quickly transferred to centrifuge tubes and swung at 8000 revolutions for 5 minutes. The clear serum was pipetted off and a portion was tested at once (No. 1); the balance was then kept at room temperature, tests being made at intervals of 15 minutes. The time from the start of bleeding

to the beginning of centrifugation was 25 minutes, and the first test was made at 52 minutes.

It will be seen that of the 13 tests made during the 3 hours following the centrifugation, only the first was fatal. The experiment might therefore be interpreted as showing that a rapid decrease occurred in the centrifugated serum. The validity of such conclusion, however, is doubtful, owing to the absence of a parallel control series, similar to that of Table 113, made with the same blood. Several other trials have shown that the serum obtained under conditions similar to those in the above experiment retained its toxicity for an hour. Still more pertinent is the fact shown in Table 106, No. 12, that the toxicity may persist for 22 hours. In this respect, therefore, the primary toxicity of serum is like that of anaphylatoxin.

Comparative Stability of Serum Toxicity at 37 C., and at Room Temperature.—Mita and Ito³² claim that a decrease in the toxicity of normal rabbit serum usually makes its appearance in from 1½ to 4 hours after bleeding. This decrease at times was found to amount to 50%. We have observed that rod sera which killed in 6 c.c. dose in a subacute manner were innocuous in like dose when tested 6 hours later, but the results in general are not very convincing. To interpret a single failure to kill, or even 2 or 3 such negative results, as a permanent decrease in toxicity is not justifiable. It must ever be borne in mind that the guinea-pig presents extreme variations in its susceptibility to anaphylatoxin, and the same holds true for the primary toxicity of a serum. The fact that the first animal of a series is killed, as for example No. 1, Table 114, while the next 12 recover, is far from being evidence of a decrease in toxicity. The more reasonable view is that the fatal case is due to the chance employment of a very susceptible animal; it will be observed in the table mentioned that 3 other guinea-pigs experienced severe shocks. In other words, the amount of poison, in the dose of serum used, was practically sublethal.

Examples of the extreme variation in guinea-pigs have been presented in Tables 45 and 71, and identical conditions exist as to normal sera. The test animal is, therefore, a greater variant than the poison. It is certain that the primary toxicity may persist in a given serum for many hours. Thus, in 3 instances, we found the sera to be just as toxic in dose of 6 c.c. after being kept at room temperature for 22 to 27 hours as they were in the beginning; an instance of this is given in Table 106, test No. 12. Again, as the result of many observations, it

may be said that when a rod serum, in dose of 3 or 6 c.c., causes several deaths during the first hour, it will be found to be equally toxic when retested on a like number of animals at the end of 6 hours, the serum being kept at room temperature.

It was of interest to learn whether a given toxic serum would become less active when kept at 37 C. than the same material held at room temperature. An experiment planned with this object in mind is given in Table 115. A rabbit was bled as usual into a carotid pipet ($3\frac{1}{2}$ minutes), and the blood was blown into a bead flask previously iced; after 4 minutes, when clotting began, it was swung for 1 minute. The blood was then centrifugated at 8000 revolutions for 5 minutes,

TABLE 115

COMPARATIVE TOXICITY OF SERUM FROM ONE RABBIT: CHILLED BEAD DEFIBRINATION; PORTION A WAS KEPT AT ROOM TEMPERATURE; PORTION B, AT 37 C.

Exper.	Guinea-Pig		Serum		Result
	Number	Weight	Intravenous Injection (c.c.)	Interval (hr.)	
A	1	182	3	—	2:55"
	2	178	"	$\frac{1}{4}$	3:30"
	3	201	"	$\frac{1}{2}$	Near-kill
	4	200	"	$\frac{3}{4}$	3:15"
	5	185	"	1	Very slight
	6	195	"	$1\frac{1}{4}$	Slight
	7	202	"	$1\frac{1}{2}$	Nil
B	8	173	"	$\frac{1}{4}$	2:55"
	9	181	"	$\frac{1}{2}$	Very severe
	10	181	"	$\frac{3}{4}$	Slight
	11	203	"	1	"
	12	205	"	$1\frac{1}{4}$	Nil
	13	210	"	$1\frac{1}{2}$	Very severe

The interval from start of bleeding to the first injection was 40 minutes; the other tests followed at times stated.

and the resulting serum was divided into 2 portions: one portion (A) was tested at once (No. 1), and the balance of the serum was kept in the room and retested at intervals; the other portion (B) was placed at 37 C., and likewise tested at 15 minute intervals.

The results given in Table 115 are also to be seen in Chart XV. It would appear that the toxicity in both portions dropped after a while, that at 37 C. seeming to be more marked. It should be noted, however, that with portion A, excluding No. 1 for purpose of comparison, there were 3 slight and 3 fatal or nearly fatal shocks, while with portion B there were also obtained 3 slight and 3 very severe or fatal shocks.

The difference was such as to hardly warrant the conclusion which at first suggested itself.

The results would have been more striking if a pair of tests were made at each interval with each serum. This would serve to bring out, as it has done in the work with anaphylatoxin, the error of placing undue emphasis upon the results of single tests.

Toxicity of Defibrinated Blood Obtained with Heated Beads.—According to Moldovan,¹¹ defibrinated rabbit blood though fatal to rabbits was rarely so to guinea-pigs. It is difficult to understand this statement, since in our work the guinea-pig proved to be very susceptible. Similarly, exception must be taken to his assertion that rabbit blood is without effect when injected before coagulation. Tables 96 to

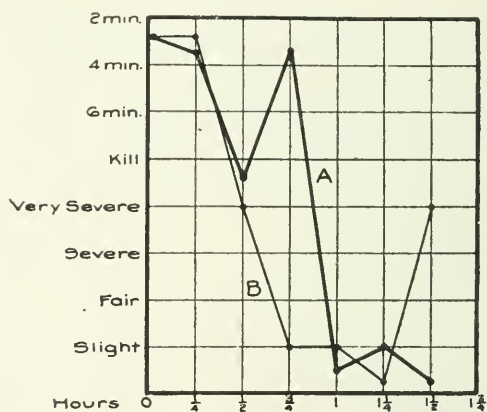


Chart 15. Comparative toxicity of normal rabbit serum: A, in room; B, at 37 C. (Table 115).

98 present sufficient evidence on this point. Of special importance is his view that the toxicity of serum and of defibrinated blood is labile at room temperature, and that exposure to 56 C. for 10 minutes results in its rapid disappearance, since large doses were without effect. It has been shown that toxicity persists at room temperature and even at 37 C.; it was in order next to determine the effect of a higher degree of heat. As is well known, anaphylatoxin is not readily destroyed at 60 C., and if the statement mentioned was correct, it would indicate that there was an essential difference between the poison of defibrinated blood and the former.

When rabbit blood is defibrinated with chilled beads, and is allowed to remain in contact with the clot and beads at room temperature, it

will be found to be acutely fatal in dose of 3 c.c., and this toxicity may persist for an hour, the duration of the experiment.

It has been pointed out that bead defibrination of rabbit blood at 40 C. seems to yield a relatively weak poison; this suggested the possibility of utilizing this method as a means of producing rabbit serum of a minimal toxicity. With this in mind, the bloods of 6 rabbits were tested. The freshly drawn blood was blown into bead flasks, previously warmed in a water-bath to 40 or 42 C., and defibrinated at this temperature, after which it was kept in the room and tested at intervals. The defibrinated blood thus obtained was found to be practically non-

TABLE 116

PRODUCTION OF TOXICITY IN RABBIT BLOOD: BEAD DEFIBRINATED AND KEPT AT 55 C. (A); AND AT 60 C. (B, C)

Exper.	Guinea-Pig		Blood		Result
	Number	Weight	Intravenous Injection (c.c.)	Interval* (min.)	
A	1	202	3	19	Very severe
	2	205	"	34	Severe
	3	201	4	50	4'50"
	4	202	"	73	Fair
	5	192	"	84	Good
	6	198	"	99	3'
B	7	222	7	15	2'35"
	8	205	5	18	2'15"
	9	210	3	22	Very severe
	10	202	5	30	2'10"
O	11	210	5	14	3'45"
	12	205	3	21	Near-kill
	13	210	3	31	7'30"

* The interval represents the time from start of bleeding to the injection.

toxic in doses up to 7 c.c., though in one instance 5 c.c. proved fatal. As a rule, 10 c.c., representing 6 c.c. of serum, were fatal, causing either acute or more often very delayed deaths (up to 72 minutes). Even 12 c.c. could be given without effect in some animals, though usually it was fatal. A striking fact in connection with these tests was that the toxicity persisted in these defibrinated bloods throughout the duration of the experiments, $\frac{3}{4}$ to $1\frac{1}{4}$ hours.

These results led to similar experiments, in which the defibrination was effected in bead flasks, previously warmed to 45, 50, 55, and 60 C., after which the blood was kept at room temperature. Here again the

bloods were found to be acutely toxic in dose of from 5 to 10 c.c., smaller amounts not being tested. The toxicity persisted through the duration of the experiments, up to 45 minutes.

Inasmuch as the blood in all of these experiments was kept at room temperature, it was deemed important to ascertain whether the toxicity persisted at 55 or 60 C. Three experiments of this kind are given in Table 116. For these the blood was drawn into carotid pipets and at once blown into bead flasks, kept in a water-bath at 55 or 60 C.; the defibrination was effected in the bath, and the flasks were left there.

The results given in Table 116 demonstrate that the primary toxicity of rabbit blood was not destroyed in 1 hour 40 minutes at 55 C., or in half an hour at 60 C. It was not possible to make additional tests, since in both experiments at 60 C. the blood was coagulated solid in 45 minutes.

Even 3 c.c. of the blood, representing 1.8 c.c. of serum, may be fatally toxic (No. 13). It must not be inferred that the poison was made at these high temperatures. As pointed out in connection with Table 44 (see also Part IX), it is more likely that it is produced while the temperature is rising and before it passes much beyond 50 C. An exposure for a short time at 50 C. suffices to inactivate the matrix of anaphylatoxin.

THE TOXICITY OF NORMAL GUINEA-PIG BLOOD AND SERUM

In investigating the toxicity of the blood and serum of the guinea-pig, one is confronted with difficulties which are not encountered in that of the rabbit. The size of the animals is such as to preclude the obtaining of a large amount of blood from one animal. Consequently, if a series of tests is to be made, a pool of the bloods of several animals must be employed. This is objectionable, since individual variations in the blood source cannot be taken into account, and especially because one cannot measure accurately the time relations of coagulation and toxicity. The first series of experiments performed were analogous to those given in Table 96. They established the fact that guinea-pig blood, like that of the rabbit, is not toxic at the moment of withdrawal, but develops toxicity in the pre-coagulation period.

Toxicity of Fresh Undefibrinated Guinea-Pig Blood.—Each test given in Table 117, represents a separate transfusion. A single donor served for Nos. 1 and 2; another was used for Nos. 3 and 4; and still another for Nos. 5 to 9. For each test the blood was drawn by punc-

ture through the thoracic wall; the blood was received into a syringe previously rinsed in salt solution. It was then kept in the syringe for different intervals of time, and finally injected into guinea-pigs.

Three cubic centimeters of such blood was not sufficient to produce a serious effect, even when held in the syringe for from 3 to 3½ minutes. This dose of rabbit blood, under these conditions, gave either a severe or a fatal shock. It is noteworthy that in Nos. 3 and 4 clotting had already started in the syringe when the injections were made. This was apparent by the pressure that had to be exerted to make the injections; and yet there was but little effect produced. The injection of clot per se, with its accompanying thrombin, is therefore not the cause of the severe effects which may follow an injection of this blood.

TABLE 117
TRANSFUSION OF NORMAL GUINEA-PIG BLOOD TO GUINEA-PIGS

Recipient			Interval*	Result
Number	Weight	Blood Transferred (c.c.)		
1	205	3	1'	Nil
2	240	"	2'	"
3	184	"	3'23"	Very slight
4	195	"	4'	"
5	185	"	3'	Fair
6	185	5	30"	Nil
7	182	"	2'	Slight
8	178	"	3'	5'50"
9	210	"	3'	8'30"

* This is the time from end of bleeding to end of injection, i. e., time in syringe. The drawing of the blood required from 10 to 30 seconds, as did also the injection.

When a dose of 5 c.c. of blood is used, the result is different. Here also, injections made at short intervals (Nos. 6 and 7) produced little effect, just as in the case of a corresponding test with rabbit blood (Table 96, No. 8). With intervals of 3 minutes, however, the 5 c.c. dose killed with typical symptoms of shock. The animal was excited, breathed rapidly, then became dyspneic, developed spasms, severe convulsions, was thrown on its side, and died with the typical agonal reflex. The autopsy findings in all of the fatal cases resulting from the injection of guinea-pig blood or serum were typical of anaphylatoxic poisoning.

It will be seen, therefore, that homologous blood in the case of the guinea-pig is distinctly less toxic than the heterologous rat or rabbit

blood. A blood may be toxic not only to the homologous animal, but even to the donor, though we have made no direct tests with the latter.

Toxicity of Normal Guinea-Pig Serum: Rod Defibrination.—For the experiment given in Table 118, 5 guinea-pigs were bled as rapidly as possible. The Noyy heart pipet (Fig. 1) was employed in drawing the blood. The animals were anesthetized and bled from the exposed heart. Rod defibrination was carried out by an assistant. The total time of bleeding, from the start of the first to the end of the last bleed, was $12\frac{1}{2}$ minutes. The blood from the 5 pipets was then drawn up into a bulb pipet and transferred at once to 2 large centrifuge tubes. These were whirled at 8000 revolutions for 2 minutes, after which the serum was pooled. Of this pooled, clear serum, 6 c.c. were tested at once; the remainder was placed at 38 C. and tested at intervals of 15 minutes.

TABLE 118
APPARENT VARIATION IN TOXICITY OF NORMAL GUINEA-PIG SERUM, KEPT AT 38 C.: ROD DEFIBRINATION

Guinea-Pig		Serum		Result
Number	Weight	Intravenous Injection (c.c.)	Time at 38 C. (hr.)	
1	200	6	—	Slight
2	170	"	$\frac{1}{4}$	$\frac{3}{40}$ Typical shock and autopsy
3	190	"	$\frac{1}{2}$	Very slight
4	180	"	$\frac{3}{4}$	Nil
5	170	"	1	"

The first injection was made 20 minutes after the start of the first bleeding; the other injections are reckoned from this point.

An apparent variation in the toxicity of the serum, like that in Table 101, is to be noted. This does not mean that the amount of the poison rose and fell during the time immediately preceding and following test No. 2, but rather it indicates that the animal in the fatal test was more susceptible than the average.

It will be seen, further, that the killing dose of a normal guinea-pig serum may not be much higher than that of many samples of normal rabbit serum. The smallest dose of normal rabbit serum that we have been able to kill with is 2 c.c. (Tables 199 and 110) and 1 c.c. (Table 97). Differences between the toxicity of normal rabbit and guinea-pig sera, however, do exist, since out of a given number of trials, the serum of the latter shows a much lower percentage of severe shocks and deaths than does that of the former species.

Toxicity of Normal Guinea-Pig Serum after Spontaneous Clotting.

—The previous work had shown that the toxicity of a serum was affected by the mode of defibrination, and that spontaneous clotting gave distinctly more toxic sera than such as were obtained by means of the rod. It was therefore of interest to see if guinea-pig serum would be influenced in like manner.

For Exper. A, Table 119, 5 guinea-pigs were bled in rapid succession, the blood being drawn into a heart pipet, and at once blown into a large centrifuge tube. The time from the start of the first to the end

TABLE 119

EFFECT OF SPONTANEOUS CLOTTING ON TOXICITY OF GUINEA-PIG SERUM: CONTACT WITH CLOT FOR 20 MINUTES (A); VARIABLE CONTACT (B AND C)

Exper.	Guinea-Pig		Serum			Result
	Number	Weight	Intravenous Injection (c.c.)	Time at Room Temperature	Clot Contact*	
A	1	202	3	—	20'	Slight
	2	193	6	5'	"	Very slight
	3	201	"	22'	"	23'15"
	4	182	"	35'	"	Severe
B	5	208	6	24'	5'	Nil
	6	175	"	20'	10'	7'35"
	7	199	"	35'	15'	Slight
	8	203	"	40'	20'	"
	9	210	"	47'	30'	"
	10	198	"	1 hr. 22'	1 hr.	3'15"
	11	196	"	18 hr. 45"	18 hr. 30'	3'40"
C	12	182	6	30'	10'	Good
	13	181	"	1 hr. 19'	1 hr.	20'
	14	201	"	18 " 34'	18 hr. 15'	Nil
	14a	181	"	" " "	" " "	Slight

* By contact time is designated the interval from start of bleeding to beginning of centrifugation at 8000 revolutions, i. e., time of contact with clot.

The injection of No. 1 was made 1 hour after the start of bleeding; the injections of Nos. 2 to 4 are reckoned from this point, the same pooled serum being used.

Nos. 5 to 14 were each made with the serum of a single donor, and the time given represents the interval from the start of bleeding to end of injection.

of the last bleed was 6 minutes. Within 20 minutes after starting to bleed, this pooled, spontaneously coagulated blood was centrifuging at 8000 revolutions, and this was continued for 15 minutes. The resulting pooled serum was then pipetted off and tested at once in dose of 3 c.c. (No. 1); the remainder of the serum was kept at room temperature and tested at intervals shown in the table (Nos. 2 to 4).

Of 3 tests with dose of 6 c.c., one was fatal. The shock in this case resembled in a striking manner (nasal foam), the slow death

occurring with feebly toxic rabbit serum. The autopsy findings confirmed the resemblance shown in the symptoms complex; lungs in maximal distention, peppered with hemorrhages and edematous; heart beating and clot absent.

Although the time of contact of clot with the serum in Exper. A of Table 119 was but 20 minutes, the injection of No. 1 was not made till 1 hour after starting to bleed, this delay being chiefly due to the long time required for the high speed centrifuge to come to a rest. The result was not very satisfactory; consequently, a series of tests were planned, with varying clot contact, to determine the effect upon the toxicity of the serum.

Exper. B of Table 119 represents such a series of tests. For these tests, 7 guinea-pigs were bled from the heart as usual, and the blood of each animal was blown at once into separate centrifuge tubes which were then kept undisturbed for different intervals of time at room temperature. They were then centrifuged at 8000 revolutions for 5 minutes, the machine being brought to rest as soon as possible by applying the brake. Each serum was then tested with the result shown in the table.

Of the 7 tests, 3 gave acutely fatal shocks which were typical; as in Test No. 3, more or less foam flowed from the nose, and on autopsy the distended lungs were found to be peppered with hemorrhages and edematous, the heart was beating, and free of clot.

The success of these tests led to a repetition of Nos. 6, 10, and 11 on the following day (Exper. C). For this purpose, 3 guinea-pigs were bled, and the bloods were treated as before. The serum used for Nos. 14 and 14a was very lipoidal; this fact may have something to do with the result. Of the 3 sera thus tested, only 1 proved fatal; the autopsy in this case, made 14 minutes after death, showed a slight auricular heart beat, no clot, lungs distended and very edematous.

In all of these tests the greatest precautions to insure sterility were taken to avoid the possibility of the formation of a bacterial anaphylatoxin. The sera remained perfectly sterile.

The few workers who have dealt with the toxicity of normal guinea-pig serum have laid stress upon the idea that it disappears rapidly after coagulation. Slatinéano and Ciuca²⁶ tested such serum 30 minutes after bleeding and found that it produced slight symptoms of shock in guinea-pigs in dose of 5 c.c.; severe effects in 10 c.c. dose, and that it killed typically when 13 c.c. were injected. Tested 24 hours

later, the serum had become innocuous, as evidenced by a single trial; without doubt, further tests would have shown that toxicity was still present. As shown in test No. 11, a guinea-pig serum kept in contact with its clot for 18 hours is toxic in dose of 6 c.c.

In contrast to the results of the above-named investigators, it is to be noted that a lethal dose of 6 c.c. can be easily obtained. We have shown that this can be done, either by rod defibrination, or by allowing the serum to remain for some time in contact with the clot. Particularly noteworthy is the fact of variation in toxicity of the sera of different animals. Thus, in Expts. B and C where each test represents a different serum, it will be seen that with like dosage only 4 of 10 sera were toxic. This difference in results, however, may be due in part to the variable resistance of the test animals, since evidence of such variation has been presented heretofore.

Toxicity of Normal Guinea-Pig Serum: Bead Defibrination.—In view of the effect of beads on the toxicity of rabbit serum, it was desirable to see if like results could be obtained with guinea-pig serum. The coagulation time of rabbit serum is much easier to control than that of the guinea-pig; the mere icing of the flask containing beads is not enough to retard noticeably the clotting of the latter blood. By packing the bead flask in a freezing mixture at -4 to $-9^{\circ}\text{C}.$, coagulation can be somewhat retarded.

For the few attempts made by this method, the guinea-pig was bled in the usual way and the blood was blown at once into a bead flask, previously packed in a freezing mixture of salt and cracked ice at $-9^{\circ}\text{C}.$ The flask was then kept at room temperature; in 2 minutes the blood had congealed, but on slight shaking it became liquid. In another 2 minutes, when coagulation did set in, the flask was shaken for 30 seconds; the blood was then decanted into a centrifuge tube and whirled at 3000 revolutions for 5 minutes. The resulting serum was tested on 2 guinea-pigs. No. 1 (187 gm.) received 2 c.c., 18 minutes after the start of bleed; at once severe dyspnea and spasms occurred. In No. 2 (205 gm.) 2.5 c.c. gave a near-kill: severe dyspnea, convulsions, thrown in 1 minute, complete suppression of respiration, but eventual recovery.

These experiments were not carried further, but they indicate that with appropriate methods one can obtain a normal serum, which, even in small dose, is by no means harmless for the homologous animal. It is possible that 3 c.c. of the serum used in the preceding experiments would have proven fatal.

The observation of Wassermann and Keysser³⁶ that pooled normal guinea-pig serum could be toxic in 4, 3 and even 2 c.c. is deserving of mention at this point. Assuming that due care was taken to exclude anaphylatoxin-production by bacterial contamination, and that the recipients were not abnormally weak, there remain 2 possible causes for this unusual toxicity.

It has been shown that normal rabbits may possess a blood which is fatal on transfusion in dose of 2 and even 1 c.c., and that the serum from such animal may be also toxic in dose of 1 c.c. (Table 97). From such observations it might be assumed that normal guinea-pigs will show a like variation. Without doubt, the blood of every species of animal is subject to changes of this kind but the limits of such variation are different and depend upon the susceptibility of the animal. Guinea-pigs are very susceptible, but they are not all equally so. This is seen in the fact that with a given dose of poison half of the animals in a series may die while the other half show no effect. But it is reasonable to believe that with 2 or 3 multiples of such dose all of the test animals will die. It follows, in other words, that the guinea-pig, unlike the rabbit, cannot be a carrier of more than 1 or 2 average lethal doses of poison per 200 gm. of body-weight. On this basis, it might be possible to obtain a guinea-pig serum which would be initially toxic in 5 c.c. dose, but it would be difficult to account thus for the 2 c.c. dose. Hence a variable inherent toxicity of the blood cannot account for the highly poisonous, pooled serum. Rather the cause must be looked for in the method of getting the blood, especially in coagulation conditions. The latter being favorable, it is possible to obtain a serum which will be toxic in 3 c.c. dose.

Toxicity of Guinea-Pig Blood: Rod Defibrination.—Some experiments to show the comparative effects of rod and bead defibrination on the toxicity of guinea-pig blood were also carried out. The blood was used in preference to serum because it was possible to make the injections much sooner after bleeding, the time consumed in centrifugation being eliminated.

A number of tests were made with blood defibrinated with the glass rod; of these, 3 are given in series A of Table 120. They serve to show that such whipped blood is not very toxic, since 5 c.c. can be given without much effect. As shown in Table 99, No. 2, 3 c.c. of defibrinated rabbit blood may be acutely fatal.

³⁶ Folia serol., 1911, 7, p. 243; Ztschr. f. Hyg. u. Infektionskr., 1911, 68, pp. 541, 544; Centralbl. f. Bakteriöl., 1, Ref., 1911, 50, Beiheft, pp. 52, 72, 78.

For the tests mentioned, 3 guinea-pigs were bled into heart pipets and each blood was at once whipped with the rod. The bloods were defibrinated for 5, 3, and 2 minutes, respectively, and tested in the order given on guinea-pigs Nos. 1, 2, and 3. The injections were made as rapidly as possible after the completion of the whipping of each blood, yet notwithstanding this fact, the bloods proved to be rather harmless.

Toxicity of Guinea-Pig Blood: Chilled Bead Defibrination.—In view of these unpromising results, bead defibrination was next resorted to, and rather striking results were obtained, as will be seen from

TABLE 120

PRODUCTION OF TOXICITY IN GUINEA-PIG BLOOD: ROD DEFIBRINATED (A); CHILLED BEADS (B)

Series	Exper.	Guinea-Pig		Blood		Result
		Number	Weight	Intravenous Injection (c.c.)	Interval*	
A	1	1	184	5	6'	Slight
		2	207	"	3'45"	Fair
		3	191	"	3'	Very slight
B	2	4	193	3	14'	46'
		5	201	4	17'	Fair
	3	6	178	3	10'	23'
		7	175	4	17'	30'
	4	8	177	3	11'	Very slight
		9	212	5	26'	Fair
	5	10	181	6	6'	2'20"
		11	179	5	14'	3'20"
	6	12	210	3	10'	Near-kill
		13	200	4	18'	6'10"
		14	190	3 (serum)	20'	Very slight
	7	15	198	3	12'	Nil
		16	205	4	28'	Very severe
		17	180	3 (serum)	16'	" slight

* The interval (Nos. 1 to 3) indicates the time from end of bleeding to end of injection; for the others, it is time from the start of bleeding to end of injection.

Series B of Table 120. They are not as marked, however, as those with rabbit blood. Furthermore, it must be emphasized that the fatal effects obtained in these experiments with small doses of defibrinated blood, are not necessarily due to the contained serum. Thus, the death following the injection of 3 c.c. of the blood does not mean that 1.5 c.c. of the serum would be fatal. This is clear from the results of Expts. 6 and 7, where tests were made with the blood and also with the serum.

For Expers. 2, 3, and 4 the bead flasks were previously chilled by being placed in a freezing mixture of salt and cracked ice at about -4°C . Large guinea-pigs were bled into heart pipets, and the blood of each was at once blown into a corresponding chilled bead flask, which was then kept at room temperature. In Exper. 2 the blood began to show a surface coagulation in 6 minutes; it was then swung gently for 2 minutes, but this was not sufficient, since a secondary clot formed; hence it was again gently agitated for half a minute. The retarding effect of the cold beads, however, was such that a third clotting developed, but this was disposed of by swinging for 10 seconds. This blood in dose of 3 c.c. caused a delayed death (No. 4).

In Exper. 3 the coagulation also began in 6 minutes; after agitation for 2 minutes, the blood was at once injected into Nos. 6 and 7, with fatal results.

In Exper. 4 a firm clot developed in the flask in 2 minutes. This was vigorously shaken for 2 minutes, but the fibrin failed to collect on the beads; this necessitated filtering the blood through cotton. The results were mild, probably due to the rapid coagulation of the blood.

For Exper. 5 the bead flask was chilled outside the window at about -10°C . for 10 minutes. After addition of the guinea-pig blood, the contents were given at once a gentle swing for 4 minutes at room temperature. A portion was then tested and, inasmuch as it gave a very acute death, which might be interpreted as due to partially unclotted blood, the remainder was filtered through cotton and injected into No. 11. This likewise caused a typical acute fatal shock. The autopsies were made 9 and 4 minutes, respectively, after death and were typical: maximal distention of lungs, heart beating, no clot. Three c.c. of such defibrinated blood, representing 1.8 c.c. of serum or less, should be fatal for the homologous animal. The toxic effects, however, must not be ascribed wholly to the serum present, but rather to a catalyzing substance which induces further poison production in the recipient, as already pointed out.

Another experiment similar to No. 5 was performed at the same time. The bead flask was cooled as before, and after adding the blood, it was at once gently swung for 6 minutes at room temperature, after which it was set aside for another 6 minutes. This blood, tested 13 minutes after end of bleed in dose of 3 c.c., caused death in about 4 hours; 4 c.c. produced a good shock, but the animal recovered.

In still another experiment, in which the bead flask was not chilled, after defibrination for 3 minutes at room temperature, a test with

2.5 c.c. was made 6 minutes after start of bleed and caused typical shock and death in 5'30"; the autopsy performed 10 minutes after death was likewise typical: the heart still beating, absence of clot, and maximal distention of lungs. The same blood left in contact with the beads was retested 12 minutes later, and in 3 c.c. dose it caused death in 3 hours.

Of five other bloods, tested in like manner, unchilled beads being used, the defibrination ranging from 2 to 6 minutes, only one caused deaths which were delayed for about 3 hours.

In 2 other experiments, which were made at the same time as Exper. 5, but with unchilled beads, the defibrination being 3 minutes. 5 c.c. of one and 6 c.c. of the other injected 4 and 8 minutes, respectively, after the end of bleeding, gave only moderate shock effects.

For Exper. 6 an old female in advanced pregnancy was employed. The blood was received into a chilled flask and kept in the room for 3 minutes when it was gently swung for 1 minute. It will be noted that 3 c.c. gave a near-kill, while 4 c.c., caused death in 6'10". The serum from this blood, however, in 3 c.c. dose produced only a very slight effect. In Exper. 7 the blood and serum of a nonpregnant female were tested in the same way, and while 4 c.c. of the former produced a very severe shock, the serum was again without much effect. A similar result as regards the toxicity of blood and serum was noted in another experiment with blood from an animal in advanced pregnancy. These results show that the poisonous effect of defibrinated blood is not wholly due to the soluble poison contained in the serum, but that an additional factor is involved.

These various results are mentioned for the purpose of bringing out the fact that the individual bloods appear to vary in their poison production during coagulation, just as guinea-pig serum of different origin varies in the ease and extent to which it gives rise to anaphylatoxin, when treated with agar or other inducing agents. Incidentally, they show that defibrination with unchilled beads does not give as high a percentage of toxic bloods as is obtained when thoroughly chilled beads are employed.

THE TOXICITY OF NORMAL RAT SERUM

This serum, because of the extreme ease with which it can be toxified by alien substances, is of special interest. The influence of the different methods of defibrination on the toxicity has not been investigated. We have observed, however, that mere incubation of sterile rat

serum for a long time, 12 to 24 or more hours, often renders it toxic, so that 1 c.c. is fatal to a guinea-pig of 200 gm. Fresh normal rat serum, when obtained by the ordinary method of rod defibrination, is rarely harmful in dose of 5 to 6 c.c. It is needful, then, only to incubate such serum, at about 37 C., to disturb in some manner its equilibrium and thereby bring about poison production. While the sera of rabbits and guinea-pigs can be made quite toxic by certain methods of defibrination, we have never noticed that mere incubation exerts so great an action upon their toxicity.

In toxicity tests with rat serum, as with all other sera, it is necessary to remember that two variable factors are involved in the experiment. One lies in the individuality of the donor which may be seen in the ease or difficulty with which its serum may be toxified; the other

TABLE 121
APPARENT VARIATION IN THE TOXICITY OF RAT SERUM KEPT AT 37 C.

Guinea-Pig		Serum		Result
Number	Weight	Intravenous Injection (c.c.)	Time at 37 C. (hr.)	
1	195	4	—	Nil
2	185	"	1/4	4'40"
3	179	"	1/2	Slight
4	190	"	3/4	Moderate
5	201	"	1	4'5"
6	204	"	1 1/4	Fair
7	166	"	1 1/2	Slight
8	193	"	1 3/4	3'50"

The first injection was made 1 1/4 hr. after the start of the first bleeding.

appears in a like peculiarity of the recipient and is recognized as a variable resistance on the part of the animal to the poison. This latter factor is well brought out in Table 121.

For the experiment there given, 10 normal rats were bled into heart pipets. Each blood was defibrinated separately by means of the rod. The blood was then pooled and centrifuged at 3000 revolutions for one hour. A portion of the serum thus obtained was tested at once (No. 1); the balance was placed at 37 C. and tested at intervals of 15 minutes. The results of these tests are shown in the table and in the Chart 16. It will be seen that of the 8 tests, 3 resulted in acute death. The symptoms were typical of anaphylactic shock, as also were the autopsy findings, the examinations being made 3 minutes after death.

It might be supposed that the poison fluctuated in amount, but it has been firmly established that such is not the case. The observed variation must be ascribed to the varying resistance of the test animals. In this experiment the short duration of incubation probably has no effect upon the toxicity; it follows then that the poison was present in the pooled serum as soon as prepared. Once developed, it persists for a long period of time, the same as in any anaphylatoxic serum.

Lastly, as in the case of other sera, the toxicity of rat serum is influenced by clot contact. In connection with another experiment (page 533), a pooled serum was prepared and tested. The blood from each animal was blown from the heart pipet into a common cylinder. The blood of 8 rats was thus collected in half an hour. The coagulated

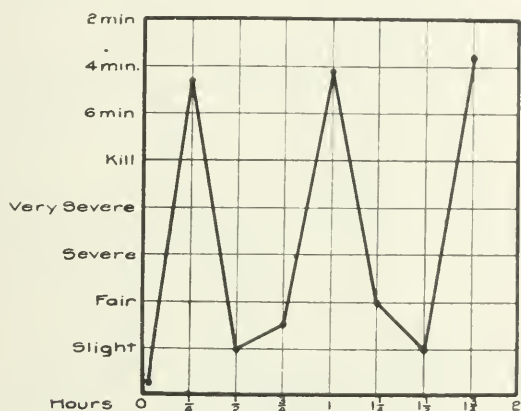


Chart 16. Apparent variation in toxicity of normal rat serum (Table 121).

mass was then defibrinated with a glass rod, and the blood centrifuged at 3000 revolutions. The serum was set aside at 0 C. for $5\frac{1}{2}$ hours before it was tested; consequently, the injections were made about $6\frac{1}{4}$ hours after start of bleeding.

A guinea-pig of 170 gm. which received 4 c.c. of this serum responded with a typical shock and death in 4'30". Another of 185 gm. was given 3 c.c. with the same result, death occurring in 3'30". The injection of 2 c.c. gave but a slight effect. It will be seen, therefore, that the toxicity of normal rat serum can be easily doubled by mere clot contact. In view of the great lability of rat serum, it should be possible to toxify it by such contact to the same extent as in case of rabbit serum.

SUMMARY

Very rapid transfusion of the blood of normal rabbit to the guinea-pig or white rat shows that the toxicity of such blood is subject to great variation. It is often possible to transfuse 10 c.c. of heart blood without much effect. Exceptionally, however, the blood from apparently normal rabbits is inherently toxic in dose of 2 or even 1 c.c., and the plasma and serum from such blood may be correspondingly toxic (Table 97).

The natural resistance of the rabbit enables it to act as a carrier of the poison which may be generated in the normal animal as a result of changed conditions, notably diet. The toxicity of the blood of an apparently normal rabbit may correspond to 100 guinea-pig lethal doses per kilo.

A blood which is initially nontoxic becomes poisonous in 3 c.c. dose, just prior to the appearance of coagulation. The speed of poison-production corresponds to that of anaphylatoxin by agar, inulin, trypanosomes, etc.

The defibrinated rabbit blood may be toxic in dose of 2 to 3 c.c., the toxicity of the preclot stage being maintained for some time.

This precoagulation toxicity, as well as that of defibrinated blood, and the inherent toxicity of normal rabbit blood may be due in part to the presence of preformed anaphylatoxin; it is probably due, chiefly, to the formation of something which acts as an accelerator or catalyzer within the recipient, thereby producing poison, *in vivo*.

This is indicated by the fact that the serum obtained from such bloods is less toxic than the poisonous blood from which it is obtained; also, by the fact that the transfusion of blood from rats injected with toxic blood may be fatal to guinea-pigs, whereas like transfusions after injection of toxic serum are negative.

Further, the rat, like the guinea-pig, is subject to acute fatal shock when injected with any one of the above mentioned 3 types of toxic bloods. In view of the great insusceptibility of the rat to anaphylatoxin, it follows that the cause of death is not the anaphylatoxin which may have been present in the injected blood; it shows that another factor is involved, an inducing agent or accelerator which acts like agar, peptone, etc.

The presence of an accelerator in preclot blood is demonstrable by injection into white rats; their blood on being transfused to guinea-pigs may cause death (Table 100). As in similar experiments with agar and peptone, a period of incubation is necessary to develop toxicity in

the recipient of the rabbit blood. Similar injections of rats with large amounts of highly toxic rabbit serum do not give evidence, on subsequent transfusion, of *in vivo* production of poison.

It is to be inferred that changes in the blood, in the preclot stage, give rise to a catalyzing agent which acts in the same way as alien substances (agar, etc.), and causes the production, *in vitro*, of some anaphylatoxin; the latter persists in the clear serum and is responsible for its primary toxicity.

Poison production and fibrin formation are parallel phenomena induced by the presence of accelerating or catalyzing substances. In each, the matrix is to be considered as undergoing an intramolecular change resulting in tautomeric modifications.

The serum is not as active as the original poisonous blood. This seeming decrease in toxicity is moderate when the serum is obtained by centrifugation at 3000 revolutions; it appears to be more marked when prepared by swinging at 8000 revolutions (Table 98).

The decrease reaches its lowest point in about 45 minutes, after which the toxicity persists unchanged for a long time (22 to 27 hours, Table 106); it is not affected by keeping at 37 C. (Table 115). This permanent toxicity of a serum is due to the soluble anaphylatoxin, while that which quickly disappears is probably due to some residual accelerator.

The decrease is not in evidence in defibrinated blood. The defibrinated blood obtained with beads, at 40 C., is relatively less toxic than such as is obtained at room temperature. On the other hand, blood which is defibrinated with beads, at 55 C., is toxic in 4 c.c. dose, and remains so; similarly, defibrinated at 60 C., the toxicity persists for the duration of the experiment (Table 116). The poison is not destroyed at such temperatures.

The toxicity of rabbit serum is influenced greatly by the conditions under which defibrination is affected. Rod defibrination gives the least toxic serum, one which kills in 6 c.c. dose. Spontaneous coagulation, with short or long contact with clot, appears to yield less toxicity than with contact of 20 minutes, the lethal dose of the latter being 2 to 3 c.c. (Table 106).

The most active serum is obtained by bead defibrination, but the temperature by influencing the speed of coagulation is of moment. Bead defibrination at 40 C. gives less toxicity than when carried out at room temperature (Table 108), or at 0 C. (Table 112). The chilling

of beads and flask, thereby retarding the coagulation, gives the best result: 2 to 3 c.c. lethal dose (Table 109). The chilled rod method appears to give no better result than the unchilled. The addition of blood which is on the point of coagulation to the chilled bead flask does not yield a very toxic serum (Table 111). Contact of serum with clot gives maximal toxicity (Table 113).

The apparent variation in the toxicity of normal rabbit, rat, or guinea-pig serum is not due to a change in the poison, but to the varying resistance of the guinea-pigs (Tables 101, 118, 121). The toxicity of the sera, apart from methods employed in defibrination, vary with the individual animal.

The symptoms and autopsy findings in guinea-pigs, after injection of toxic blood or serum, are essentially those produced by anaphylatoxin, and in anaphylactic shock. Identical results are obtained when rat or guinea-pig blood or serum is used instead of that of the rabbit. The homologous blood is distinctly less toxic than heterologous.

Large doses of normal rabbit serum which kill guinea-pigs rapidly cause death with thrombi in the heart and large vessels. This may occur with serum which is more than 24 hours old. Smaller doses kill, but at autopsy no thrombi are to be found (Tables 103, 104). The clot observed at autopsy is probably always of postmortem origin and cannot, therefore, be considered as the cause of death.

Strikingly different pictures are to be found at autopsy of animals dying from large doses of serum, depending upon whether death occurs rapidly (2 to 4 minutes), or more slowly (5 minutes to 2 hours). The findings in guinea-pigs succumbing subacutely to homologous serum resemble closely those of a slow killing rabbit serum; with acute ending they correspond to those of typical anaphylactic shock.

Sodium oxalate, when added to rabbit serum, and allowed to act for a short time, will prevent thrombosis, but will not protect against a fatal issue; a longer contact, however, may render the serum non-toxic. Similarly, the addition of sodium oxalate to preclot blood will prevent not only coagulation, but also poison-production. The recalcification of an oxalate plasma results in development of toxicity as well as coagulation (Table 105).

Guinea-pig blood, like that of the rabbit and rat, is not toxic at the moment of withdrawal, but becomes so in the preclot stage. Under like conditions, guinea-pig blood is not as toxic to a guinea-pig as is rabbit blood (Table 117). When partially clotted, it may be injected in 3 c.c. dose without effect.

On rod defibrination, guinea-pig blood may yield a serum which is toxic in dose of 6 c.c.; a like result is obtained in spontaneous clotting (Table 119). With chilled beads, it is possible to get a serum which will kill in 3 c.c. dose.

The defibrinated blood of guinea-pig, prepared with rod, is less toxic than that obtained with chilled beads; the latter may be toxic in 3 c.c. dose. As in case of rabbit blood, this toxicity is not entirely due to the poison in solution, since the serum from such blood is less toxic than its source (Table 120).

Normal rat blood may become toxic by prolonged incubation. While the serum obtained from a single rat, by rod defibrination is rarely fatal in 6 c.c. dose, it is possible, by clot contact, to increase the toxicity so that 3 c.c. will kill.

The speed of poison production in blood in the preclot stage, the effects in animals, the resistance of rats to the toxic serum, the persistence of the toxicity at room temperature, at 37 C., and especially at 55 to 60 C., indicate that anaphylatoxin is developed in normal bloods before and during coagulation. The normal toxicity of serum is, therefore, to be correlated with anaphylatoxin; the latter is produced by changes incidental to clotting; it is increased in amount when the serum is subsequently brought into contact with alien substances, such as trypanosomes, bacteria, agar, inulin, etc.

IX. SPECIFIC ANAPHYLACTIC SHOCK

F. G. NOVY AND P. H. DEKRUIF

SYNOPSIS

INTRODUCTION

SPECIFIC ANAPHYLACTIC SHOCK IN SENSITIZED RATS; INSUSCEPTIBILITY OF THE RAT

NONSPECIFIC SHOCK IN SENSITIZED RATS; THE SPIRILLAR PARADOX
RATS SENSITIZED TO EGG-WHITE; ALSO NORMAL RATS

EFFECT OF VERY DILUTE EGG-WHITE; OF DISTILLED WATER

DISTILLED WATER BUT NOT SALT SOLUTION PRODUCES

FATAL ANAPHYLACTIC SHOCK IN SENSITIZED RATS

RATS SENSITIZED TO HORSE SERUM

EFFECT OF VERY DILUTE HORSE SERUM; OF DISTILLED
WATER

NONSPECIFIC SHOCK IN GUINEA-PIGS

DISTILLED WATER EQUALLY FATAL TO SENSITIZED AND TO NORMAL GUINEA-PIGS

TOLERATION FOR SALT SOLUTION

THE IN-VIVO PRODUCTION OF POISON

IN SHOCKED GUINEA-PIGS, SENSITIZED TO EGG-WHITE; TO
HORSE SERUM

IN RATS SENSITIZED TO EGG-WHITE; TO HORSE SERUM

THE IN-VITRO PRODUCTION OF POISON

ACTION OF EGG-WHITE ON SERUM OF RAT SENSITIZED TO
EGG-WHITE

ITS ACTION ON NORMAL RAT SERUM

ACTION OF DISTILLED WATER ON SERUM OF RAT SENSITIZED
TO EGG-WHITE

ITS ACTION ON NORMAL RAT SERUM, HEATED AND
UNHEATED

ACTION OF HORSE SERUM ON THAT OF RAT SENSITIZED TO
HORSE SERUM

ACTION OF DISTILLED WATER ON SERUM OF RAT SENSITIZED
TO HORSE SERUM

ACTION OF RABBIT, BEEF AND GUINEA-PIG SERUM ON CORRESPONDING SENSITIZED RAT SERA

NONCOAGULABILITY OF BLOOD IN SHOCKED GUINEA-PIGS

SUMMARY

For the study of this phenomenon, the rat was elected as first choice for the reason that previous experience had shown that it exhibited 2 very important characteristics which, though fully discussed heretofore, may be briefly mentioned.

In the first place, its serum was found to yield a most powerful anaphylatoxin. Under proper conditions, as has been shown, it is

possible to render normal rat serum toxic, within 15 minutes, so that from 0.2 to 0.25 c.c. will kill a 200 gm. guinea-pig on intravenous injection. With inulin, it has been possible to reduce the lethal dose to 0.15 c.c. (Part X). When it is recalled that the primary toxicity of normal rat serum is such that it only rarely kills guinea-pigs in dose of 4 or 5 c.c., it will be seen that this means, at least, a 20 or 30 fold increase in its poisonous property. The sera of rabbit and guinea-pig, under like conditions, give at best only a threefold to sixfold increase in toxicity. It is obvious, therefore, that the rat serum is decidedly more labile than the sera mentioned, and it seemed reasonable to believe that the plasma in corpore would share this characteristic.

In the second place, it was found that the rat was insusceptible to enormous doses of anaphylatoxin. Thus, of a given anaphylatoxin which killed a 247 gm. guinea-pig in dose of 0.25 c.c., as much as 15 c.c. were injected into a 151 gm. white rat without causing death, though the shock was severe (Table 58). The rat, therefore, compared with the guinea-pig, possesses a remarkable tolerance as to this poison. At first sight, it would seem that this resistance was cellular in character, an adaptation or off-set, as it were, to the highly labile plasma of the animal. But, it is possible that the real explanation will be found elsewhere, perhaps in purely physical reactions, reversible in character. The fact remains, however, that, weight for weight, the rat will tolerate more than 100 times the amount of poison necessary to kill a guinea-pig.

In view of the foregoing acts, it would seem that in anaphylactic shock the guinea-pig may develop relatively but little poison, since the production of a single fatal dose is sufficient to cause death. At most, one could expect but a few lethal doses since a guinea-pig serum does not yield a powerful poison; it is possible, with agar, to render it toxic in 1 c.c. dose (Part II). Assuming, for example, that a 200 gm. guinea-pig has 12 c.c. plasma, it would seem that, at most, 12 lethal doses could be expected, supposing that the plasma was as toxifiable as the serum. However, it may be more reactive, owing to the presence of the labile fibrinogen.

On the other hand, in the rat because of its tolerance for the poison, an anaphylactic shock if induced should be the result of the formation of a large amount of poison, probably in excess of 100 guinea-pig lethal doses. It may be questioned whether such a large amount of

poison could be produced within the rat. Thus, assuming as above that a 200 gm. rat has 12 c.c. plasma, it would be capable of yielding 80 guinea-pig lethal doses, each being 0.15 c.c. This calculation would show that unless the fibrinogen, or perhaps the cell plasma, reacted and thus increased the amount of poison, it would not be possible to produce a fatal specific anaphylactic shock in the rat. These considerations show that the blood of the rat should be better adapted than that of the guinea-pig for the study of anaphylactic reaction. The rat proved to be a most useful reagent in this study.

SPECIFIC ANAPHYLACTIC SHOCK IN THE SENSITIZED RAT

In order to test their behavior to specific shock, a large number of rats were sensitized by the intravenous injection of diverse sera and also egg-white. Rather unexpectedly, it was found that the rat did not respond, certainly not fatally, to the second injection. An examination of the literature failed to add any light, for, apparently, but a few workers have attempted to induce anaphylactic shock in rats.

The first mention of tests with this species was made by Arthus¹ who stated that he had observed anaphylactic phenomena in guinea-pigs and rats after repeated injections of horse serum, and that the results would be described later. It has not been possible to find, in his subsequent papers, any further allusion to this matter. Rosenau and Anderson² mention having tested rats, among other animals, but the work was to be reported on at a later date. Uhlenhuth and Haendel³ did not succeed in producing anaphylaxis in mice and rats; and Trommsdorff,⁴ repeating the work in Uhlenhuth's laboratory, sensitized rats and mice to egg-white but in neither could he induce symptoms, much less fatal anaphylactic shock. The behavior of mice to anaphylactic shock has been studied by many workers with varying results. Of these, Braun,⁵ Ritz⁶ and also Sarnowski⁷ succeeded. It is therefore possible that given special conditions, even rats may be made to respond. According to Ritz and Sachs,⁸ the mouse, while

¹ Compt. rend. Soc. de biol., 1903, 55, p. 819.

² Bull. No. 29, Hyg. Lab. U. S. P. H. S., 1906, p. 57.

³ Centralbl. f. Bakteriologie, I, Ref., 1910, 47, Beiheft, p. 68.

⁴ Arb. a. d. K. Gsndtsamte, 1909, 32, p. 567. Centralbl. f. Bakteriologie, I, Ref., 1909, 44, Beiheft, p. 152.

⁵ München. med. Wchnschr., 1909, 56, p. 1880. Ztschr. f. Immunitätsf., 1909, 4, p. 600.

⁶ Ztschr. f. Immunitätsf., 1911, 9, p. 331. Centralbl. f. Bakteriologie, I, Ref., 1910, 47, Beiheft, p. 63.

⁷ Ztschr. f. Immunitätsf., 1913, 17, p. 577.

⁸ Centralbl. f. Bakteriologie, I, Ref., 1911, 50, Beiheft, p. 45.

insusceptible to anaphylatoxin, reacts with peptone and to specific anaphylactic shock.

The greater part of the tests on rats were made either with horse serum or egg-white, and the results are of sufficient interest to be given at length. Tables 122 and 123 embody the results obtained with horse serum; in the first of these tables, the rats received only a single sensitizing injection of 0.01 c.c. of the serum (1 c.c. of 1:100). When retested after a suitable interval, the rats showed practically no effects except when the antigen was given in large dose (Nos. 5 and 6).

Since Sarnowski's work on mouse anaphylaxis had shown that a single injection of a small amount of serum did not sensitize as well as a large amount, it seemed likely that the nonfatal, and even mild

TABLE 122

ANAPHYLACTIC REACTION IN RATS SENSITIZED WITH SINGLE SMALL DOSE OF HORSE SERUM

Rat		Serum (c.c.)		Result
No.	Weight	First Inject.	Second Inject.	
1	150	0.01	0.5 on 13th day	Nil
2	156	"	1 " " "	"
3	148	"	2 " " "	Very irregular respiration
4	158	"	1 " 52nd "	Slight respiration trouble
5	168	"	3 " " "	Severe shock; irregular respiration, slight spasms, severe dyspnea, depression
6	150	"	3 " " "	Very severe dyspnea, spasms, in 3 minutes apnea. Used for transfusion
7C	155		2	Deep rapid respiration

All injections intravenous. Nos. 4 and 5 were reinjected as Nos. 1 and 2 of Table 123. No. 7 was a normal rat and served as a control.

results given in Table 122, were due to a similar condition. Accordingly, sensitization, with several varying doses, was resorted to. In Table 123 will be found the results of such tests on 14 rats. It will be seen that nearly all of the test animals showed more or less respiratory disturbance, at times some dyspnea and slight jerky spasms, but at no time was a fatal result produced. As indicated in the foot-note to the table, 4 of the rats were later retested with a fourth injection with no particular increase in the symptoms.

It will be observed that rats which received 2 or 3 preliminary injections did not react much more than those that had but one (Table 122). These results clearly show that the rat does not respond with severe anaphylactic shock, in the manner of the guinea-pig. The remarkable tolerance of the rat to anaphylatoxin, which has been pointed out heretofore, finds further corroboration in these experi-

ments, since not enough of the poison is formed in the sensitized rat to produce death. That there is a partial reaction, there can be no doubt, judging from the symptoms; further proof will be given later on, when it will be shown that in rats thus treated there is an actual in-vivo production of poison.

The results obtained with different antigens, such as the sera of guinea-pig, rabbit, beef, and with solutions of egg-white, were essentially the same as those presented above. The incompleteness of the anaphylactic reaction when an undiluted antigen was used, as was the case in Tables 122, and 123, suggested the possibility that a better result might be obtained with one that was diluted. The basis for this

TABLE 123
ANAPHYLACTIC REACTION IN RATS SENSITIZED WITH MULTIPLE DOSES OF HORSE SERUM

Rat		Serum (c.c.)		Result
No.	Weight	Preparation	Last Injection*	
1	135	Jan. 3, 0.01; Feb. 24, 1.0	3 on 16 day	Respiration, deep, jerky
2	160	" " " " 3.0	" " "	Severe dyspnea
3	152	" " Jan. 16, 2.0	1 " 39 "	Slow jerky respiration. No spasms
4	150	" " " " 1.0	" " " "	Short severe dyspnea
5	110	" " " " 0.5	" " " "	Slight spasms
6	165	Dec. 30, 0.01; Jan. 17, 0.5	" " 38 "	" " Deep respiration
7	190	" " " " " "	3 " " "	No spasms. Deep respiration
8	125	" " " " " "	" " " "	Some dyspnea. Deep respiration
9	150	" " " " " "	" " " "	Slightly irregular respiration
10	146	" " " " " "	" " " "	Rapid deep respiration
11	135	" " " " " "	" " " "	Nil
12	124	" " " " " "	" " " "	Nil
13	115	" " " " " "	" " " "	Deep respiration
14	123	" " " " " "	" " " "	Very deep respiration

* The time given refers to the number of days since the last sensitizing dose was given. All injections, intravenous. Nos. 3, 6, 9 and 10 were each given a fourth injection, 16 days later, of 3 c.c. Marked peripheral irritation, irregular respiration to slight dyspnea, with light jerky spasms were noted in the first 2; the effects in the last 2 were much less severe.

belief will be given further on. Accordingly, 2 such tests were made with an antigen which was diluted nineteenfold (Table 124, Nos. 6 and 7). In the first of these, with dilute beef serum (No. 6), the result was distinctly more severe than the companion test where 4 c.c. of the undiluted serum were given, this amount being 16 times that given No. 6. In this test the serum was diluted with salt solution.

For the second test with diluted antigen, a rat sensitized to rabbit serum was used; the dilution was effected with distilled water instead of salt solution. It will be noted that the outcome was a protracted death, such as has been usually observed in mice. This was the first instance of fatal anaphylactic shock in the rat.

In the tests with guinea-pig serum, Nos. 1 to 3, Table 124, only a moderate respiratory disturbance was produced, corresponding to that seen in the work with horse serum. It is of interest to observe that beef serum, which is relatively toxic to a guinea-pig, produced very little effect, in the control normal rat No. 4, in dose of 4 c.c. This is in keeping with the tolerance for anaphylatoxin which the rat has been shown to possess, and it suggests the desirability of studying the behavior of the rat to various highly toxic sera, such as those of eel, man, etc. In the sensitized rat, the beef serum caused a fair shock,

TABLE 124
ANAPHYLACTIC REACTION IN RATS SENSITIZED WITH DIFFERENT ANTIGENS

Antigen	Rat		Antigen (c.c.) Given on		Result
	No.	Weight	First Inject.	Second Inject.	
Guinea-pig Serum	1	145	0.25	1 on 17 day	Some dyspnea. Very deep respiration
	2	125	"	2 " " "	Very irregular deep respiration
	3	140	"	3 " " "	" " " respiration
Beef serum	4 C	150	0.25	4 " on 16 day	Respiration shallow, interrupted
	5	140		" " " " *	Fair shock. In 10 min. severe peripheral irritation. Resp. irregular
	6	105		5 " " " "	Severe shock. Excited. Deep resp.
Rabbit Serum	7	127	"	5 " " " †	2 hrs. 15 min. Excited. Very rapid respiration, then slow and jerky
Egg-white‡	8	120	1	1 " " "	Very excited. Very rapid respiration, later slow and deep
	9	125	"	5 " " "	Slow, labored respiration

* 0.25 c.c. beef serum diluted with 4.75 c.c. salt solution.

† 0.25 c.c. rabbit serum diluted with 4.75 c.c. distilled water.

‡ This was a 1% solution of Kahlbaum's desiccated egg-white, dissolved in 0.85% salt. All injections were intravenous.

but a considerably greater effect followed the use of the highly diluted antigen (No. 6). The result with the similarly diluted rabbit serum (No. 7) was heuristic and opened up an interesting line of study. The egg-white being more alien to an animal than a serum could be expected to give a greater reaction; this, however, was not the case and the explanation is that a more concentrated solution, corresponding in protein content to a serum, would have been probably better than a 1 per cent. solution. Likewise, the use of high dilutions of egg-white with distilled water, similar to the dose in Nos. 6 and 7, will be found desirable (Table 125).

NONSPECIFIC ANAPHYLACTIC SHOCK IN SENSITIZED RATS

The foregoing experiments show quite conclusively that the antigen, when injected by itself into a sensitized rat, is incapable of releasing a fatal shock under conditions which would have met with a prompt response in a guinea-pig. Some indication of a reaction, however, was in evidence and this led to the belief that a slight alteration in the state of the blood plasma might yield a better result. A basis for such view is to be found in the known fact that undiluted sera often fail to give specific reactions, whereas when diluted they are instantly effective (Neisser and Wechsberg⁹).

In particular, we were guided by an unpublished observation made some years ago by one of us (Novy) concerning the paradoxical action of hyperimmune rat blood on relapsing spirochetes. This serum was tested undiluted, as well as in serial dilution, up to as high as 1:1000, for its agglutination, spirillicidal, and lytic reactions. The action of the highly diluted serum was immediate and striking; the spirochetes on contact with such dilutions instantly showed intense hyperexcitability, then rapidly agglutinated in enormous stellate masses, after which the motion soon decreased, then disappeared; the now motionless spirochetes each developed one or more globular swellings (plasmoptysic in character and not buds as supposed by some); this process rapidly went on to full lysis so that in a few minutes nothing but a mass of minute granules was left. In brief, the diluted hyperimmune serum induced an intense in-vitro Pfeiffer's phenomenon.

In very low dilutions, especially in undiluted serum, the reaction was different. At first, there was hyperexcitability, then moderate agglutination, accompanied by a gradual decrease in motility up to a certain point; then, of a sudden, the unagglutinated cells began to show a quickening motion, and this revival extended to all of the organisms, the spirochetes disengaged themselves from the loosely agglutinated masses and became free and active just as if it were a normal serum. This paradoxical behavior of the diluted, versus the undiluted serum is connected with a further observation where rats immunized by 30 or more injections of spirillar blood, showed active spirochetes in their circulation, almost continuously during the hyperimmune stage. Pfeiffer's isolation of the live cholera vibrio from the peritoneal cavity of the hyperimmune guinea-pig is of like meaning.

⁹ München. med. Wchnschr., 1901, 48, p. 697.

Both reactions are obviously of the Neisser-Wechsberg type; they show clearly that while antibodies are present in such immune blood or serum, they are unable to act either on account of the aggregation or concentration of the constituents, or because of some hindering or calyptic substance. Parenthetically, it may be added that the cause of "fastness" in an organism may be sought in the reciprocal aggregation of its protoplasmic colloids to a disturbed state in the host. Bearing in mind the view expressed, experiments were planned to test the action of highly diluted antigen upon the sensitized rat. The first 2 tests of this kind, given in Table 124, led to further trials with large amounts of fluid, which will now be considered.

TABLE 125
THE EFFECT OF DILUTE ANTIGEN (1:14) ON RATS SENSITIZED TO EGG-WHITE

Rat		Antigen (c.c.) Given on			Result
No.	Weight	First Inject. (undil.)	Second Inject. (diluted 1:14)	Dose per 100 gm.	
1	215	1	15 on 12 day	7.0	Very rapid fluttering respiration, later, interrupted and deep. Muscles leg twitch. No spasms
2	205	"	" " 17 "	7.3	Apnea while injecting. Slow return of respiration. Dyspnea. Muscle twitch
3	154	"	" " " "	9.8	Same as No. 2
4	168	"	" " " "	8.9	2'30". Apnea while injecting. No return of respiration, muscle twitch
5	198	"	" " " "	7.6	Same as No. 2*
6	185	"	" " 18 "	8.1	Immediate respiratory trouble. Dyspnea*
7	150	"	" " " "	10	Respiration stops while injecting*

The time required to inject 15 c.c. ranged from $\frac{3}{4}$ to $1\frac{1}{4}$ minute.

* The rats thus designated were bled for transfusion experiments in Table 134. All injections were intravenous.

Rats sensitized to egg-white.—For the experiments given in Table 125, the antigen, which was a 1% solution of egg-white in salt solution, was diluted with 14 parts of distilled water, and the test dose of this mixture was 15 c.c. The result was marked for, even while injecting, the respiration became fluttering and ceased entirely; in some it returned, in others it did not. Rapid muscular twitching of the injected leg, especially about the point of injection, was nearly always observed; this may be interpreted as a local cellular anaphylactic response to the dilute antigen.

The rats were sensitized by an intravenous injection of 1 c.c. of the 1% egg-white solution and were used 12 to 18 days later. Three of the rats mentioned in Table 125 were opened, while in shock, and blood was drawn from the heart and injected into guinea-pigs for

the purpose of demonstrating the in-vivo production of poison (Table 134); in all of these animals, the characteristic anaphylactic findings were noted: maximal lung distention, and drop in blood pressure. The heart of No. 2, examined within 3 minutes after the injection, was found to contain clot and much fluid blood; a similar condition was noted in No. 4; in No. 5 there was no sign of clot in the heart, even 6 minutes after death; in No. 7, notwithstanding the large volume injected, the drop in blood pressure was such that 3 c.c. of blood could be drawn with difficulty; in the heart of No. 6, when examined 5 minutes after exposure, clot was found, but this was clearly post-mortem, since the blood drawn for transfusion, at once after injection,

TABLE 126
THE EFFECT ON NORMAL RATS OF DILUTE EGG-WHITE (1:14), A; DISTILLED WATER, B

Exper.	Rat		Fluid Injected		Result
	No.	Weight	Amount (c.c.)	Dose per 100 gm.	
A Egg-White	1	145	15	10.3	Deep respiration, otherwise nil
	2	165	10	6.0	Same
	3	159	15	9.4	"
	4	206	"	7.3	" *
	5	186	"	8.0	" *
	6	210	"	7.1	" *
B Distilled Water	7	162	"	9.3	"
	8	158	12	7.6	"
	9	160	12.1	7.5	"
	10	200	15	"	"
	11	175	13.1	"	"

* The rats thus designated were bled for transfusion experiments in Table 134. The time taken for injections was $\frac{3}{4}$ - $1\frac{1}{4}$ min.

was perfectly fluid. This tendency to form clot in the heart of the sensitized animal shows a highly labilized condition of the plasma proteins, which will be brought out again in connection with Table 127.

The severe results obtained in the foregoing experiments raised the question as to whether they were not due to the mere injection of so large an amount of fluid. Controls were, therefore, made at once with normal rats. Two series of control tests are given in Table 126; of these, Series A was made with the very dilute egg-white, the same as that used in Table 125, while Series B was made with plain distilled water.

In regard to Series A, the normal rat shows an entirely different behavior from that of the sensitized one; practically no effect followed

the injection of these amounts of diluted antigen. These tests, it may be added, were made on the same day as those of Table 125.

As a further control, normal rats were injected with distilled water (Series B); the results of these tests are also to be found in Table 126. It is to be noted that distilled water may be injected intravenously in large dose without effect.

Since the normal rat was found to be practically unaffected by large doses of diluted antigen, or distilled water, it was necessary as a further control, in order to establish the specific nature of the shock observed in sensitized rats, to test the effect of a large volume of dis-

TABLE 127

THE EFFECT OF DISTILLED WATER (A), AND OF SALT SOLUTION (B), ON RATS SENSITIZED TO EGG-WHITE

Exper.	Rat		Fluid Injected		Result
	No.	Weight	Amount (c.c.)	Dose per 100 gm.	
A Distilled Water	1	163	12.2	7.5	Dyspnea and apnea while injecting. Muscle twitching. No recovery
	2	175	13.1	"	Same as No. 1
	3	160	12.1	"	" " "
	4	175	8.75	5.0	Very near-kill. Dyspnea, convulsions thrown, recovers in 10 min.
	5	198	10	"	5. Apnea while injecting. Convulsive jerks. Muscle twitching
	6	195	"	"	Practically nil
	7	175	8.75	"	Nil
B Salt Solu- tion	8	210	15.75	7.5	Practically nil
	9	196	14.7	"	" "
	10	140	10.5	"	Some respiratory trouble toward end injection. Depressed. Few jerky spasms

All injections were intravenous. The injection time was usually from 50 to 60 seconds.

tilled water in such rats. This experiment led to a surprising result. It revealed the fact that the shock results seen after the injection of the diluted antigen were not due to the latter but in reality were due to the volume of water used. In other words, the sensitized rat, unlike the normal one, responds to the injection of distilled water with a shock which can only be interpreted as anaphylactic. The reaction is considered, therefore, to be a nonspecific anaphylaxis, since the antigen is not necessary to discharge the shock in a sensitized rat.

The results of experiments in which varying amounts of distilled water were injected into sensitized rats are given in Table 127. The

rats were all sensitized by the intravenous injection of 1 c.c. of the 1% egg-white solution, and were tested on the 15th and 16th days. The controls for this set of tests are to be found in Table 126, Series B.

Autopsy, made about 3 minutes after the injection, showed in Nos. 1, 2, 3, and 5 partial lung distention and clot in the heart. Clotting was also observed in connection with tests of Table 125.

The results presented in Table 127 show that rats sensitized to egg-white are almost instantly killed by the injection of distilled water, when given in dose of 7.5 c.c. per 100 gm. body-weight; 5 c.c. per 100 gm. is less certain in its effects and may even be nil. The individual variation of the rat must be considered. On the other hand, distilled water in dose of 7.5 gm. or even more per 100 gm. of normal rat is innocuous (Table 126). These results are paralleled by those obtained with rats sensitized to horse serum.

Extremely interesting are the results obtained in tests Nos. 8 to 10 of Table 127 where the rats were sensitized in the same way as the others, and retested at the same time with salt solution (0.85%). By contrast with Nos. 1 to 3, the salt solution was practically harmless, whereas the distilled water tests were fatal. Some effect was obtained in No. 10, but this may have been due in part to the fact that the animal was rather weak, if not sickly when injected.

It may also be noticed that the results given in Table 127 are decidedly more severe than those in Table 125 where sensitized rats were given the same volume of the diluted egg-white. At first, it might seem that this difference was due to the small amount of salt injected with the egg-white. It is more likely, however, that the observed difference is due to the experimental conditions. In the tests of Table 125, the rats were etherized, fixed, and then allowed to recover, after which they were injected, while those of Table 127 were injected directly.

It is evident that the sensitized rat is different from the normal one. Though it does not respond like the sensitized guinea-pig to an antigen, nevertheless, the physical state of its plasma proteins must be greatly altered. It no longer possesses the resistance or tolerance of the normal rat, and the mere injection of distilled water, it may be assumed, produces an intense disruption of the colloidal equilibrium, resulting in intramolecular changes which yield on the one hand, anaphylatoxin, and on the other, increased rapidity of coagulation, or fibrin. The immediate cause of death must be sought in the produc-

tion of anaphylatoxin through tautomeric modification of a matrix present in the plasma, if not in the cytoplasm. The rapid formation of clot in the blood of the shocked animal is not the cause of death since the clot is not present if the heart is examined at once after death. A somewhat similar condition arises when a large dose of rabbit serum is injected into a guinea-pig. As shown in Part VIII, small doses of this serum may kill without any clot appearing in the heart, whereas with large doses, coagulation is observed, at autopsy.

It has been pointed out on page 777 that a rate of 200 gm. with 12 c.c. of plasma, is capable of developing from 50 to 80 guinea-pig lethal doses, and since it has a natural tolerance for 100 of such doses, the fatal results obtained are rather paradoxical. The first explanation for this discrepancy is that the tissue cells of the sensitized rat have become more susceptible and reactive. If that were so, a sensitized rat should reveal such increased susceptibility when injected with a large multiple dose of the poison. To test this point, a rat serum was toxified by the sol-gel method so that 0.25 c.c. was the lethal dose for a 180 gm. guinea-pig. Of this anaphylatoxin, 10 c.c., representing 40 lethal doses, were injected intravenously, in 55 seconds, into a sensitized rat of 187 gm.; only a slight respiratory disturbance and depression followed. A second sensitized rat (170 gm.) was then given a mixture consisting of 7.5 c.c. of this anaphylatoxin and 8.2 c.c. of distilled water (5 per 100), to see if the presence of distilled water would aid the poison; the result in this case was perhaps a trifle more severe than in the former.

These 2 tests show that the action of the distilled water in the sensitized rat is not limited to the toxification of the plasma, but that the explosive reaction probably involves the sensitized cell plasma as well. A sensitized animal does not possess an increased susceptibility to anaphylatoxin; it can, however, produce poison more readily. Sensitization may mean a change in the cytoplasm as well as in the blood plasma.

Rats Sensitized to Horse Serum.—The results obtained with rats sensitized to horse serum correspond to those noted in connection with egg-white (Table 125). Though the tests were not as extensive as with the latter, they sufficed to show that the sensitized rat responds quickly to the injection of either dilute antigen or distilled water, whereas the control normal rats (Nos. 11 to 13) which received the same amount of the dilute antigen showed practically no effect. The

controls for the action of distilled water upon normal rats are to be found in Table 126.

The rats employed for tests Nos. 1 to 6 of Table 128 were sensitized by a single intravenous injection of 0.25 c.c. of horse serum; Nos. 4 and 5 were tested 12 days later, the others on the 20th day. Nos. 7 to 10 had previously received multiple injections of the antigen (Table 123), and were retested 30 days after the last injection, with varying concentrations of horse serum. The antigen concentration is indicated in the table. The injection time varied from $\frac{3}{4}$ to $1\frac{1}{2}$ minute, except in case of No. 7 which took $2\frac{1}{2}$ minutes; recovery was probably due to this fact.

TABLE 128
THE EFFECT OF DILUTE ANTIGEN IN RATS SENSITIZED TO HORSE SERUM

Exper.	Rat		Antigen			Result
	No.	Weight	Dilution	Amount (c.c.)	Dose per 100 gm.	
A	1	149	1+14	15	10	1'30". Dyspnea, spasms, convulsions
	2	?	"	"	—	45". Instant apnea
	3	158	"	"	9.5	2". While injecting dyspnea, then apnea; followed by prompt return of respiration, then peripheral irritation, dyspnea, spasms, convulsions
	4	175	"	"	8.6	Marked respiratory effects. Died during night
	5	180	1+9	10	5.5	Nil
	6	?	"	"	—	Slight respiratory trouble
B	7	135	1+19	10	7.4	Respiration suppressed but returns
	8	141	"	"	7.1	1'45". Respiration suppressed while injecting
	9	152	1+39	"	6.6	Respiration suppressed but recovers
	10	150	1+59	15	10	1'40". Respiration suppressed. Muscle twitching
C Control	11	156	1+14	15	9.6	Nil
	12	170	"	"	8.8	"
	13	152	"	"	9.9	"

Nos. 11 to 13 are normal rats which were injected with the dilute antigen as controls.

It will be seen that the dilute antigen in dose of 7 c.c. or more per 100 gm. is usually fatal to sensitized rats. That this volume of liquid of itself is not responsible for the effects observed will be seen from the control tests Nos. 11 to 13. The effects produced are the same as those seen in connection with the tests of rats sensitized to egg-white. Autopsy usually showed permanent maximal lung distention. In the 5 acute deaths of Tables 128, the heart, examined within 3 minutes after death, showed much clot; it is possible that in one of these the change was premortal, but of that there can be no certainty.

Since the dilute antigen has a marked action upon rats sensitized to horse serum, it was of interest to see if distilled water would not have the same action. The experiments in Table 129 were made with this object in mind. The rats were sensitized by intravenous injections of 1 c.c. of horse serum, and were tested 14 days later. The time required to make these injections was about 1 minute.

It will be seen from the table that rats sensitized to horse serum, on injection with distilled water, behave the same as those prepared with egg-white. The dose of 7.5 c.c. per 100 gm. body-weight is acutely fatal to the sensitized rat; 5 c.c. per 100 gm. has less or no

TABLE 129
THE EFFECT OF DISTILLED WATER ON RATS SENSITIZED TO HORSE SERUM

Exper.	Rat		Fluid Injected		Result
	No.	Weight	Amount (c.c.)	Dose per 100 gm.	
A	1	175	13.1	7.5	1'30". Respiration suppressed while injecting. Jerky spasms. Muscle twitchings
	2	204	15	"	1'30". Dyspnea. Convulsions
	3	150	11.2	"	1". Apnea. Severe convulsions
	4	191	9.5	5.0	Respiration almost suppressed
	5	198	10	"	Nil
	6	180	5	2.8	"
B	7*	173	13	7.5	Respiration not suppressed, but severe depression. Died during night
	8†	175	"	"	Respiration not suppressed
C	9‡	175	"	"	Respiration suppressed, but returns very shallow. Slight spasms
Control	10‡	160	12	"	Respiration very rapid, shallow

* Received 1 c.c. horse serum + 12 c.c. salt solution.

† Received a mixture of 1 c.c. horse serum, 6 c.c. of salt solution and 6 c.c. of distilled water.

‡ These are sensitized rats which received only salt solution.

effect. It is significant that the injection of salt solution in dose of 7.5 c.c. per 100 gm. results in much less effect (Nos. 9 and 10). Even the addition of antigen to the salt solution is not enough to overcome the inhibitory action of the salt (Nos. 7 and 8). Similar results after salt injection into rats sensitized with egg-white are given in Table 127, B.

The autopsy of Nos. 1 to 3 showed partial to maximal distention of the lungs. The heart of No. 1, examined 3 minutes after death, showed a large clot; but in Nos. 2 and 3 where the examination was

made in 2 and 1 minutes, respectively, after death, no clot was present. This fact, as pointed out before (Table 127), indicates clearly that coagulation is not the immediate cause of death.

NONSPECIFIC SHOCK IN SENSITIZED GUINEA-PIGS

The peculiar behavior of sensitized rats to distilled water, on injection, yielding an intense nonspecific anaphylactic shock, made it desirable to ascertain if a similar condition could be demonstrated in sensitized guinea-pigs. With this object in view, a series of tests were made, the results of which are given in Table 130. The guinea-pigs were sensitized by intravenous injection of 1 c.c. of the 1% solution of egg-white; they were tested 12 days later (Nos. 1 to 5).

TABLE 130
THE EFFECT OF DISTILLED WATER ON GUINEA-PIGS SENSITIZED TO EGG-WHITE (A, B); ALSO
ON NORMAL GUINEA-PIGS (C)

Exper.	Guinea-Pig		Distilled Water		Injection Time	Result
	No.	Weight	Amount (c.c.)	Dose per 100 gm.		
A	1	260	13	5	1'20"	Very slight
	2	240	17	7.5	1'45"	Slight
	3	205	20	10	2'	Slight
B	4	250	12.5	5	35"	Very slight
	5	201	15	7.5	45"	32%. At once apnea, recovery. Then relapse
C Controls	6	200	10	5	1'	Nil
	7	195	14.6	7.5	1'30"	Very slight
	8	199	15	"	1'	1'40%. At once apnea
	9	203	"	7.4	1'	1'20%. " " "
	10	210	"	7.1	3'	Slight
	11	202	10	5	45"	"

It will be seen from the table that the sensitized guinea-pigs, on injection with distilled water, behave about the same as the normal animals (Nos. 6 to 11). When the speed of injection was halved, as in Exper. B., there was possibly an increased effect but, as shown in the controls (Nos. 8 and 9), this cannot be ascribed to the sensitized condition. By increasing the time of injection to 2 minutes, it was possible to inject 10 c.c. per 100 gm. without appreciable effect (No. 3). As to the speed of injection, even a fraction of a minute seemed to markedly affect the results. This was especially seen in connection with the controls where dose of 7.5 c.c. per 100 gm. was shown to be

fatal, provided it was injected within 1 minute. Thus, while Nos. 8 and 9 died rapidly under these conditions, Nos. 7 and 10, with a longer injection time, exhibited little effect. The autopsies made 3 minutes after death showed maximal distention of lungs in No. 8, and partial distention in No. 9; the hearts were not beating, and clot was present in both.

The rather indifferent results obtained in these tests suggested the possibility that the guinea-pigs were not sufficiently sensitized, but No. 4 retested with 1 c.c. of egg-white, $1\frac{3}{4}$ hours after the injection of the distilled water, promptly responded with atypical shock and died in 3'30". Again, Nos. 1 to 3 were retested, in like manner 30 hours later; No. 1 apparently showed no effect and it seemed as if desensitization had resulted from the previous injection of water; but Nos. 2 and 3 responded with typical shock, showing that the result mentioned was probably due to individual variation in resistance of the test animals.

The foregoing experiments show that the rapid injection of much distilled water is fatal to the normal guinea-pig, while the sensitized animal is not any more susceptible. By contrast, sensitized rats react more readily than normal ones, and furthermore, speed is not as important a factor. The question naturally arises as to the reason for the difference in the behavior of the sensitized rat and guinea-pig. Undoubtedly, the explanation is to be found in the relative ease with which the respective sera are toxified. Thus, under like conditions, working with an agar sol-gel, the toxic dose of the treated rat serum will be at least 6 times that of the guinea-pig, 1.5 c.c.:0.25. The rat plasma, inherently labile, is rendered more so by the process of sensitization, whereas that of the guinea-pig is not. It will be shown later (Tables 137 and 144) that sensitized rat serum is considerably more easily toxified by the action of distilled water than is normal serum. The in-vitro action of distilled water has not been tested, in like manner, on normal and sensitized guinea-pig serum. It would be of interest to do so and also to test the lability of the normal and sensitized sera as to speed of poison-production on treatment with agar sol-gel, or with inulin.

It has been pointed out in connection with Tables 127 and 129 that salt solution, in amounts corresponding to the fatal dose of distilled water, produces but moderate effects in rats. This fact also holds true for guinea-pigs, as will be seen from the results of tests given in

Table 131. The first 10 tests were made with the intent to bring out, if possible, a nonspecific shock with a dilute alien protein in normal guinea-pigs; this result was not realized, though with larger amounts of such protein it must be obtainable.

Of the 3 tests (Exper. A) made with egg-white, diluted with distilled water, one resulted in acute death, one in a fair shock and one showed very little effect. The symptoms were the same as those fol-

TABLE 131
EFFECT ON NORMAL GUINEA-PIGS OF SALT SOLUTION WITH EGG-WHITE (B); AND WITHOUT (C); A RECEIVED EGG-WHITE PLUS DISTILLED WATER

Exper.	Guinea-Pig		Fluid Injected		Injection Time	Result
	No	Weight	Amount (c.c.)	Dose per 100 gm.		
A*	1	210	15	7.2	1'	2'. At once apnea
	2	200	"	7.5	"	Fair
	3	201	"	"	"	Very slight
B†	4	195	"	7.7	"	Practically nil
	5	199	"	7.5	"	Slight
	6	226	"	6.6	"	"
	7	210	"	7.1	1'15"	1 hr. 20 min. At once apnea, recovery, then relapse
	8	218	"	6.9	1'	Slight
	9	230	"	6.5	"	Very slight
	10	233	10	4.3	45"	" "
	11	210	20	9.5	1'15"	Died during night. Severe, marked hypothermia, 25 C.
	12	206	"	9.7	"	Moderate
C‡	13	204	"	9.8	"	"
	14	200?	"	?	1'30"	Slight
	15	" ?	"	?	"	Very slight
	16	" ?	"	?	45"	"
	17	" ?	"	?	2'15"	" "

* Nos. 1, 2, and 3 received a mixture of 1 c.c. of egg-white and 14 c.c. of distilled water.

† Nos. 4 to 10 received a mixture of egg-white and salt solution; this was 2 + 8 in case of No. 10; 0.5 + 14.5 in case of No. 9; and 1 + 14 in Nos. 4 to 8.

‡ Nos. 11 to 17 were given only 0.85% salt solution; this was 37 C. in Nos. 15 to 17, and at 0 C. for Nos. 11 to 14.

The weights of Nos. 14 to 17 were not available.

lowing the injection of distilled water (Table 130); the autopsy of No. 1, made 3 minutes after death, showed maximal distention of lungs and clot in the heart.

In Exper. B, where the egg-white was diluted with salt solution, the results were markedly less severe; only one death occurred, and that after 1½ hours. Considering the dose per 100 gm. given in these tests, it is evident that the salt solution does not cause a reaction or disturbance in the blood as does distilled water.

In Exper. C, normal guinea-pigs were injected with salt solution alone, in amount corresponding to nearly 10% of the body-weight. In Nos. 11 to 14, the solution was iced before injection, while for Nos. 15 to 17, it was warmed to 37 C. The iced solution seemed to cause more pronounced response than that which was warmed to 37 C. This may have been, however, a matter of chance, since Nos. 14 and 15, which were made side by side, showed no difference. Similarly, varying the speed of the injection of the warm solution (Nos. 16 and 17) was without effect.

The guinea-pig, therefore, can tolerate salt solution much better than distilled water. It is well known that salt has an antagonistic action as regards anaphylactic shock, the action of anaphylatoxin, and peptone (Table 73).

The effects observed in normal guinea-pigs as a result of the injection of distilled water, or salt solution, are to be interpreted as of the same order as those seen in similarly treated sensitized guinea-pigs, especially in sensitized rats. The change or disturbance created in the colloids of the plasma is to be compared with the reaction induced by injections of agar, peptone, bacteria, etc., that is, it is anaphylatoxic.

THE IN-VIVO PRODUCTION OF POISON

The shock effects observed in a sensitized guinea-pig consequent on the second injection of the so-called antigen is clearly the result of a poisonous action. It may be assumed that a poison, in the sense of a chemical entity rather than as a property of a dispersed state, is produced in the reaction which follows the administration of the second dose. But it is a striking fact that such efforts as have been made to demonstrate the presence of a poison in the blood of a shocked animal have yielded little or no result. It would seem that considering the violence of the reaction, it should be possible to show that the blood was poisonous. This important result, we have been able to accomplish. As far as we know, the experiments to be related will present the first experimental proof of the in-vivo production, during anaphylactic shock, of an acutely fatal poison. This fact, taken in connection with the subsequent demonstration of the in-vitro production of the same poison, serves to throw new light upon the nature of the anaphylaxis.

It is reasonable to expect that if the poison is a chemical entity, that once made, it will persist in the body for some time. This, indeed,

is the basis of toxicologic research concerning foreign chemical poisons. On the other hand, it is possible to conceive that the enormous mass of body colloids may exert a reversive action. This, perhaps, may explain why the recovery from acute anaphylactic shock is usually rapid and complete. The blood drawn from an animal at this stage of the shock is bound to be innocuous in the case of the guinea-pig. This makes clear, in part, the failure of previous workers to demonstrate the presence of an acute poison. A further reason lies in the fact that sensitized guinea-pigs, shocked with horse serum, yield less poison, in corpore, than those treated with the strictly alien egg-white.

Guinea-Pigs Sensitized to Egg-White.—The foregoing considerations were first applied to shocked guinea-pigs, and later to rats. The former were selected at the outset, with the expectation that they would yield enough blood for 2 or 3 consecutive transfusions, and thus make it possible to show that, in a given animal, the blood was initially nontoxic, then rapidly acquired the poisonous property, and, perhaps, again lost it. This, however, was not as feasible as it would appear, owing to the fact that the rapid fall in blood pressure, in the shocked animal, limits the amount of blood which can be drawn. This difficulty may, in part, be avoided by using sensitized guinea-pigs of maximal weight. Undoubtedly, large rabbits, or even dogs, could be used to advantage for such serial transfusions but, in these animals, the rapid disappearance of the poison cannot be expected because of their greater tolerance.

For the experiment proper, the sensitized animal and one or two recipients were immobilized at the same time, on Latapie holders; the jugulars were then exposed and covered with wet cotton compress until the moment of injection. The sensitized animal was then given an intravenous injection of the antigen, in this case 2 c.c. of egg-white. The heart was at once exposed and blood drawn up into a syringe, in such amount as desired; this was then immediately injected into the first of the recipients. After an interval of from $\frac{1}{2}$ to 1 or more minutes, blood was again drawn and transferred to the second recipient.

Occasionally, some delay in drawing the blood from the heart is experienced; this may be due to plugging of the needle, but more often it is caused by the drop in blood pressure. At no time, in the following experiments, was the blood in the syringe long enough to develop

primary toxicity. As shown in Table 117, guinea-pig blood may become toxic in 5 c.c. dose, when kept in the syringe for 3 minutes. Even 6 c.c. of blood can be transfused without ill effects, provided the transfer time is kept short (Table 67).

The guinea-pigs employed in the experiments of Table 132, as donors, were sensitized by an intravenous injection of 1 c.c. of egg-white, and were tested on the 23d and 24th day with 2 c.c. of this solution. The table presents the results of 4 experiments, in each of which the donor served for 2 transfusion tests. The transfer time of the second portion was always longer than that of the first, because of the drop in blood pressure; for the same reason, the amount of blood withdrawn was less than that for the first test.

TABLE 132

THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN SENSITIZED GUINEA PIGS SHOCKED WITH EGG-WHITE

Exper.	Test No.	Weight of		Blood Trans. (c.c.)	Total Time*	Transfer Time†	Result
		Donor	Recipient				
A	1	585	206	7.0	2'40"		Practically nil 4'55". Typical shock and autopsy
	1a	"	208	3.0	4'55"		
B	2	710	210	10.0	2' 5"	1'45"	Very slight Moderate. In 7' temperature, 33.2 C.
	2a	"	206	3.0	5'55"	2'25"	
C	3	524	180	5.0	2'40"	1'40"	Moderate 3'. Typical shock
	3a	"	191	2.0	6' 3"	2'30"	
D	4	475	186	5.0	2'40"	1'40"	Slight 5'17"
	4a	"	185	3.0	5'28"	2'30"	

* Total time is that from start of injection of antigen until the end of injection of the recipient.

† Transfer time means the interval from the entrance of the syringe into the heart of the donor until its withdrawal from the recipient. It represents, therefore, the maximal time any portion of the blood was in the syringe. This transfer time must be kept as short as possible, otherwise in-vitro toxification may occur. The difference between the 2 times represents the reaction period within the donor.

The table shows that the immediate transfusion of blood, within the first 2½ minutes following the injection of the antigen, is practically without effect even when from 5 to 10 c.c. of blood are transferred. By contrast, the second transfusion of only 2 or 3 c.c. of blood gave 3 deaths and 1 moderate shock. The "total time" of such toxic blood was from 5 to 6 minutes, but the actual reaction time, in corpore, was about 3 minutes, whereas in the first transfusions it was but 1 minute.

The failure to kill in test No. 2a corresponds to like failures in transfusions, after injections of agar and peptone (See Charts 9 and

11). A similar drop in toxicity will be seen in Exper. C., Table 133. It is likely that this apparent decrease in toxicity is due to the test animal being more resistant than the average.

The autopsy findings were those of anaphylaxis. When examined 3 minutes after death, 2 showed maximal distention of lungs, vigorous heart beat, and blood perfectly fluid with no sign of clot; while 1 (No. 3a) had slightly collapsed lungs; otherwise the condition was the same as in the others. The symptoms were not those of the extreme convulsive, but rather of the intense quiet type often produced by the injection of toxic sera.

The first transfusions, made within about $2\frac{1}{2}$ minutes after the end of the injection, were practically without effect, although at this period, the shocked donor would ordinarily be in violent convulsions, indicating that at least 1 lethal dose of poison had formed in the total blood of the animal. Assuming that the blood constitutes 10% of the body-weight, this amount of poison would be distributed in from 47 to 71 c.c. of blood. The amount of blood carried over in the first transfusion from each donor was 5-7-10 c.c.; as these amounts produced but little effect, it is reasonable to conclude that the donor, at this point, had more than 1 and less than from 7 to 10 lethal doses of poison.

The fact that the second transfusions, made about 5 to 6 minutes after the injection of the antigen, were fatal in dose of from 2 to 3 c.c. would indicate that there had been a considerable increase in the amount of the poison, this increase probably continuing for some time even after apparent death. On the foregoing assumption regarding the amount of blood present, and allowing for the first transfers, it follows that donors A, C, and D had at this time 17, 23 and 14 lethal doses, respectively.

The conclusion to be drawn from these tests, therefore, is that a poison is present in the blood of the shocked guinea-pig, at about the time it would have died from the effects of shock. The symptoms and autopsy findings resulting from such transfusions are those of anaphylatoxin.

Guinea-Pigs Sensitized to Horse Serum.—The transfusion tests from sensitized guinea-pigs, after shock with egg-white, were followed by similar attempts with horse serum, the results of which are given in Table 133. The donors in these experiments were sensitized by intravenous injection of 1 c.c. of horse serum, from 23 to 25 days before the reinjection. This second injection consisted of a like amount of

serum, except in Exper. C, where the test dose was 2 c.c.; the severe effect of test No. 3 may possibly have been due to the use of this large dose, though individual susceptibility must be considered, and is indicated in the behavior of No. 3a.

These experiments showed that the guinea-pigs which were sensitized to horse serum were not as responsive to poison production as were those of the egg-white set (Table 132). After the failure to secure fatal effects in the transfusion tests (Expers. A to D), it was realized that this merely confirmed previous observations that animals which were sensitized to a strictly alien protein, such as egg-white, were more reactive than those treated with diverse sera, a fact which is especially in evidence when in-vitro experiments are made (Tables 135 and 143). While it is likely that guinea-pigs sensitized to egg-white, as pointed out above, may yield, in-vitro, from 23 to 17 lethal doses of poison, it could be assumed that those treated with horse serum gave rise to enough poison to kill the donor, not much more, perhaps only from 3 to 5 lethal doses. To detect the presence of the poison in the guinea-pig shocked with horse serum, it would require, therefore, the transfusion of a large amount of blood. With this consideration in mind, the next experiments were planned so as to increase the reaction time, and to make only one transfusion in order to get the desired large quantity of blood.

Unfortunately, the donors which were used in the subsequent experiments (E to I), were not very large; hence, it was difficult to draw much blood. This difficulty was considerable when the attempt to draw the heart blood was made after the lungs had reached maximal distention, as a result of the shock; it was this complication which, in part, caused a delay of about 3 minutes in the transfer time of No. 5. This trouble was avoided by opening up the thorax immediately after the injection of the antigen, the partial distention of the lungs interfering much less with the drawing up of the heart blood than the maximal distention. There still remained a serious obstacle which was not overcome: The great drop in blood pressure made it impossible to remove the desired amount of blood in less than 3 or 4 minutes. This accounts for the long transfer time in Expers. E to I, and the results must be interpreted with caution in view of this delayed transfusion.

It has been shown in Table 117, that 5 c.c. of normal guinea-pig blood, when held in the syringe for 3 minutes, may become fatally

toxic to the homologous animal. In the experiments under consideration, only a portion of the blood was in the syringe during the entire transfer time and as a further offset to the possible criticism that the poisonous results obtained in Nos. 5, 6, 7, and 9 were due to primary toxicity developed by this extravascular delay, it should be pointed out that shocked blood does not coagulate as rapidly as does normal blood; hence the toxic effects observed are more likely to be due to poison produced by the shock than to precoagulation changes.

TABLE 133
THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN SENSITIZED GUINEA-PIGS SHOCKED WITH HORSE SERUM

Exper.	Test No.	Weight of		Blood Trans. (c.c.)	Total Time	Transfer Time	Result
		Donor	Recipient				
A	1	550	210	5	3' 3"	2'13"	Very near-kill
B	2	650	214	10	2'18"	1'30"	Practically nil
	2a	"	213	4	3'33"		Good shock
C	3	571	205	5	1'40"	30"	" "
	3a	"	195	"	3'45"		Very slight
	3b	"	183	3	7' 3"		Fair shock
D	4	420	203	5	2'26"	1'10"	Moderate shock
	4a	"	191	2	5'15"		Slight shock
E	5	405	180	4	6'15"	3' ?	6'20"
F	6	405	179	7	4'52"	3' 7"	Near-kill
G	7	380	208	8	5'35"	3'55"	5'55"
H	8	360	198	8.5	4'50"	3'10"	Slight
I	9	435	204	9.5	6'30"	4'50"	2'30"

The blood remaining in the heart of the donors, after removal of the test portion, continued to be fluid for some time. Thus, clot was absent, and the blood in the heart was fluid in donor No. 3 when examined 13 minutes after injection; in No. 5, at 15 minutes; in No. 6, at 11 minutes; in No. 7, at 15 minutes; in No. 8, at 14 minutes. On the other hand, No. 9 showed a very slight clot at 14 minutes, a condition probably brought about by the repeated injury caused by the needle in being moved back and forth from the heart into the blood vessels. The donors of Table 132, it may be added, showed a similar delayed coagulation of the intracardial blood; Nos. 1, 2, and 4 had no sign of clot when examined at 15, 11, and 12 minutes after injection, whereas No. 3, at 15 minutes, showed a very small clot.

The autopsy findings in recipient No. 5 were typical: maximal distention of lungs, vigorous heart beat, and no clot; in No. 7, the lungs showed partial distention, but the heart beat was normal and the blood, fluid. In No. 9, however, while showing maximal distention of lungs and a good heart beat, there was a slight intracardial clot; this, however, is a condition to be expected whenever large amounts of blood or serum are injected into a guinea-pig. The relation of dose to clot effects was particularly brought out in connection with Tables 103 and 104.

In considering the results presented in Table 133, it is reasonable to conclude that they show the presence of anaphylatoxin in the blood of guinea-pigs shocked with horse serum. They confirm the results obtained with egg-white. The amount of poison was distinctly less: since a like calculation applied to Nos. 5, 7, and 9 indicates the presence of but from 5 to 10 lethal doses.

Rats Sensitized to Egg-White and to Horse Serum.—Having shown that a poison was produced, in vivo, during the anaphylactic shock of sensitized guinea-pigs, it was of interest to learn whether this was equally true for the shock observed in sensitized rats. It will be recalled that the treated rat is rather tolerant to a second injection of antigen (Tables 122 to 124), and that more pronounced results were obtained when the highly diluted antigen, or plain distilled water was injected (Tables 125, 127). Hence, for the tests given in Table 134, diluted antigen was used, except in case of Nos. 5 and 6. It would have been interesting to have made like transfusions from sensitized rats after the injection of distilled water but this was not done.

For tests Nos. 1 to 4, the rats were sensitized by intravenous injection of 1 c.c. of egg-white solution and were retested on the 18th day with a mixture consisting of 1 c.c. of the antigen and 14 c.c. of distilled water. The preliminary test, No. 1, indicated the apparent need of increasing the amount of blood transferred and decreasing the reaction time. The next experiment (No. 2), made with this object in view, seemed to be entirely successful, the reaction period being but 1'40". In test No. 3, the conditions were much the same but the amount of blood transfused was only 3 c.c., and a delayed death was obtained. Without doubt, a larger dose would have given an acutely fatal shock.

In test No. 4, the transfusion of 5 c.c. had but a very slight effect; reaction time in this instance was about the same as in Nos. 2 and 3,

and the very different result cannot be ascribed to the shorter transfer time. This rat was desensitized an hour before use, by being given an intravenous injection of dilute egg-white; as a result of this treatment, the blood did not become toxic on subsequent reinjection with dilute antigen. The desensitized rat, therefore, behaved essentially the same as the 2 controls, Nos. 5 and 6. These controls were normal rats which received injections of dilute antigen, in the same amount (15 c.c.) as the sensitized rats (Nos. 1 to 4). This injection produced no suppression of respiration, as it did in the sensitized rats, and the blood transfusion likewise showed absence of poison.

TABLE 134
THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN SENSITIZED RATS SHOCKED WITH EGG-WHITE (A), AND WITH HORSE SERUM (C)

Exper.	Test No.	Weight of		Antigen (c.c.)*	Blood Trans. (c.c.)	Total Time	Transfer Time	Result —
		Donor	Recipient					
A Egg-White	1	198	174	15	2	5'45"	24"	Slight
	2	185	201	"	5	2'23"	43"	4'30"
	3	150	212	"	3	2'35"	1'	6 hrs.
	4	117	198	"	5	2'	28"	Very slight
B Controls	5	186	203	"	4.5	2'45"	35"	Nil
	6	210	206	"	5	3' 5"	1' 5"	"
C Horse Serum	7	195	175	2	2	7'	30"	Very slight
	8	150	192	3	2	5'13"	25"	Good
	9	165	197	15	3	3'42"	1'15"	Slight
	10	123	181	"	5	2'25"	40"	Good

* Nos. 7 and 8 received the undiluted horse serum; all others were given 15 c.c. of a mixture of 1 c.c. of antigen and 14 c.c. of distilled water.

The controls in Exper. B were given the dilute egg-white solution; they were normal rats, whereas all others were sensitized.

For further controls showing the effect of transfusion of whole blood from normal rats, it will be well to consult Table 66. It will be noted therein that 4 c.c. of such blood can be transfused without appreciable effect, provided the transfer time is not much over 45 or 48 seconds; when it reaches 1 minute, this dose may cause acute death as a result of pre-coagulation toxicity. While this fact might raise some doubt as to the interpretation of the results in tests Nos. 2 and 3, it is to be remembered that the blood of these rats was greatly diluted by the injection of 15 c.c. of fluid and, hence, the transfer time for whole blood and that of such diluted blood are not directly comparable. Indeed, Control No. 6 shows that the transfer time of 65 seconds does not result in toxifying 5 c.c. of such diluted blood.

There can be no doubt, in view of these controls, that in the rat sensitized to egg-white, and subsequently shocked, the blood acquires almost at once a demonstrable poisonous property, which seemingly disappears in a few minutes (No. 1).

For tests Nos. 7 to 10, the rats were sensitized with horse serum. Nos. 9 and 10 were given 0.25 c.c. intravenously, and were retested on the 20th day with dilute antigen; No. 8 had received an injection of 0.01 c.c. and was tested on the 52nd day; while No. 7 received 2 previous injections of 0.01 and 0.5 c.c., respectively, the last being given 37 days before being used for this experiment. It will be seen that the transfusions from Nos. 8 and 10 resulted in the production of good shocks, indicating that some poison had formed in consequence of the injection of the antigen. The amount, however, was not enough to bring about a fatal shock. It has been shown, in connection with Table 133, that guinea-pigs, sensitized to horse serum, when shocked do not give rise to poison as readily, or in the same amount, as those which have been sensitized to egg-white (Table 132). A strictly alien antigen like egg-white appears to have a greater disturbing action than a serum.

The injection of undiluted horse serum into rats Nos. 7 and 8 caused severe shock effects; the hearts when examined 10 minutes later were beating vigorously and were free of clot. In the 6 sensitized rats, tested with the dilute antigen, the usual severe effects of rapid suppression of respiration were noted, and the hearts, when examined 5 minutes after the injection, were found to have more or less clot. In view of these severe effects, it is, perhaps, surprising to find that the transfused blood was not more toxic. Considering the large amount of fluid injected, the actual quantity of blood transfused was but one-half of the amount indicated; on this basis, it would appear that rat No. 2 developed only about 7 lethal doses of poison. This amount of anaphylatoxin cannot possibly account for the severe effects produced in the rat; it suggests, therefore, the desirability of further study along these lines.

THE IN-VITRO PRODUCTION OF POISON

The fact that the injection of so-called antigen, into a sensitized guinea-pig, is followed by anaphylactic shock indicates the formation, *in vivo*, of a poisonous property previously nonexistent, or an accentuation of such quality already present, though in small amount, in

the test animal. Proof of the formation of such poison, *in vivo*, during shock, has been given, but this evidence, valuable as it is, would be incomplete unless supplemented by observations showing that the same reaction, at practically the same speed, can take place in the test tube. It was logical to expect this result, provided the necessary conditions could be met, and, as will be shown, this was successfully realized.

The production of a poison, causing acute anaphylactic death, by adding antigen to sensitized serum, *in vitro*, has been attempted by various workers, notably Weichardt,¹⁰ Richet,¹¹ Rosenau and Anderson,¹² Anderson and Frost,¹³ and others with little or no success. The only one who actually succeeded in producing acute anaphylactic death was Vaughan.¹⁴ With his co-workers, he found that incubation, for 30 minutes, of a mixture of egg-white with the serum, or with the extracts of organs from a sensitized guinea-pig, yielded a poison which killed in 4 minutes; with the same mixtures, incubated for 90 minutes, the effects were less marked. Equally marked fatal results were obtained by incubating horse serum, typhoid, and cholera bacilli with the serum and extracts of homologously sensitized guinea-pigs. The failure on the part of other investigators, notably Doerr,¹⁵ to understand the significance of these results is surprising.

Since death from anaphylactic shock usually occurs within 5 minutes, it is evident that at least 1 guinea-pig lethal dose of poison is produced, *in vivo*, within this time. Assuming that the plasma constitutes 6% of the body-weight, it follows that a 200 gm. guinea-pig carries 12 c.c., which amount, therefore, in shock, would contain at least one lethal dose of the poison. If actually several of such lethal doses are developed, it will be evident that experiments, *in vitro*, may possibly succeed with one-half, or one-fourth, or even less of this amount of serum, it being assumed that the serum is as reactive as the plasma. This condition is really necessary since the injection of large amounts (from 6 to 8 c.c.) of a normal serum is likely to cause severe shock and even death, by reason of the presence of anaphylatoxin resulting from preclot changes. It has been shown that the fatal dose of normal guinea-pig serum may be 6 c.c. (Table 118); that of the

¹⁰ Deutsch. med. Wchnschr., 1902, 28, p. 625. Centralbl. f. Bakteriöl., I, Ref., 1910, 47, Beiheft, p. 36.

¹¹ Compt. rend. Soc. de biol., 1909, 66, p. 1005.

¹² Bull. 50, Hyg. Lab., U. S. P. H. S., 1909, p. 43.

¹³ Bull. 64, Hyg. Lab., U. S. P. H. S., 1910, p. 29.

¹⁴ Ztschr. f. Immunitätsf., 1911, 11, p. 675.

¹⁵ Kolle and Wassermann, Handbuch der pathogenen Mikroorganismen, 1913, 2, p. 1040.

rabbit may be also 6 and even 3 c.c. (Tables 102, 103); while that of the rat may be 4 c.c. (Table 121).

In view of this toxicity of normal sera, extreme care must be taken to avoid drawing wrong conclusions. An essential condition is the use of one and the same pooled serum for the experiment proper and for the control tests.

It will be shown that the addition of an antigen to a sensitized serum produces a poison in a very few minutes; it speeds up enormously a reaction which occurs, though at a considerably lower rate, when such antigen is added to a normal serum. The speed of the reaction is comparable to that which results when trypanosomes, bacteria, agar, peptone, inulin, etc., are added to a serum; this fact demonstrates the unity of the phenomena concerned.

Serum of Rat Sensitized to Egg-White: Action of Antigen.—The experiments in which the action of egg-white was tested on the serum of sensitized rats are given in Table 135. For Exper. A, 4 rats were bled 16 days after having been sensitized by an intravenous injection of 1 c.c. of a 1% solution of egg-white. As usual, the heart pipet method was used, and each blood was separately defibrinated by means of the rod; after centrifugation, the resulting sera were pooled (9 c.c.). The reagents, egg-white and serum, after warming for a few minutes at 37 C. in a Roux water bath, were combined in the ratio of 1:5 (1.8 + 9), thoroughly mixed, and a portion was drawn up into the syringe for immediate injection (No. 1); the balance was then returned to the water bath and tested at 4 and at 15 minutes after the mixture had been made.

No. 1, which received the mixture within 45 seconds after it had been made, responded with a slight reaction. Whether this effect was due to the action of antigen, in this short interval of time, or whether it was the effect of the primary toxicity inherent in serum, could not be determined; it is, however, unimportant. The test shows that the mixture at this early period was not particularly toxic, unless it be assumed that guinea-pig No. 1 was of the very resistant type. Had the amount of pooled serum been sufficient, it would have been well to have had control tests preceding and following the experiment; each of these controls would have received a mixture of 4 c.c. of pooled serum and 0.8 c.c. of salt solution. A control of this kind is given in connection with Exper. B (No. 6).

By contrast, test No. 2, which was made after an incubation of 4 minutes, proved fatal, showing that a marked rise in toxicity, even in that short interval had taken place. The remainder of the mixture, actually 1.4 instead of 1.2 c.c., after being heated at 37 C. for 15 minutes, was likewise fatal. The actual amount of rat serum injected was 4 and 1.2 c.c., respectively.

The experiment was repeated with like result. For the second trial, 3 rats, which had been sensitized 16 days before, were bled and each blood was defibrinated, centrifugated and the resulting sera (10 c.c.) were pooled. As before, the pooled serum was used at once for the experiment. The reagents, egg-white, pooled serum and distilled water,

TABLE 135
ACTION OF EGG-WHITE ON SENSITIZED RAT SERUM, RATIO, 1:5

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time* at 37 C.	
A	1	204	4.8	45"	Slight jerks. No dyspnea. Some posterior paralysis
	2	189	"	4' 5"	3'35". Typical shock and autopsy
	3	188	1.4	15'40"	4'25". Typical shock and autopsy
B	4	187	4.8	4'12"	4'
	5	200	2.4	5' 4"	2'15"
C Control	6	196	4.8	4'	Practically nil

* The time at 37 C. is that from moment of mixing until end of injection. The discrepancy in the total amount injected in Exper. A was due to the fact that each portion was measured by being drawn directly into the syringe.

were placed at 37 C. for a few minutes, whereupon the mixtures B and C were made, 1.2 c.c. egg-white + 6 c.c. sensitized pooled serum, and 0.8 c.c. distilled water + 4 c.c. sensitized pooled serum, respectively, and at once returned to the water bath. Nos. 4, 5, and 6 were then injected at the time indicated in the table.

Control test No. 6, in which the mixture C was used, showed no effect at a time when the antigen-serum mixture was fatal. In Experiments B and C, the same pooled serum was used and the only difference was that the former had antigen, while the latter had a corresponding amount of distilled water. The actual amount of serum injected into Nos. 4 and 5 was 4 and 2 c.c., respectively. Incubation for only 4 or

5 minutes sufficed to toxify this serum, and the minimal lethal dose was less than 2 c.c. On reference to Table 137, Nos. 10 and 13, it will be seen that 1 c.c. of a sensitized serum is fatal when incubated for 5 minutes with 6 and 9 parts of distilled water, respectively. Corresponding experiments with sensitized sera and antigens (horse, beef, rabbit, guinea-pig sera) will be found in Tables 143 and 145.

Action of Egg-White on Normal Rat Serum.—The 2 experiments in Table 135 indicate that sensitized serum on contact with its antigen, egg-white, promptly gives rise to a poison. Recognizing this fact, it is in order to inquire as to what happens when normal rat serum is incubated; first, without any addition; second, with egg-white; and third, with distilled water. The latter question will be considered on page 807.

As to the first point, it will be well to refer to Table 121, where, apparently, incubation of normal rat serum rendered it toxic in dose of 4 c.c. It must be understood that the results in this case are not strictly due to incubation; the same results would have been obtained had the serum been kept at room temperature. In that instance, the serum was primarily toxic, in dose employed, for the very susceptible individuals of the species tested, the guinea-pig. To ascertain the direct action of heat upon toxicity, it would be necessary to employ a smaller dose, making tests at hourly intervals over a long period; trials of this kind, with 2 c.c. as the dose, would be of interest, but the amount of pooled serum required for a long series of such tests is prohibitive. On a number of occasions, however, experiments of this kind were made, the dose being 1 c.c.; seemingly long incubation tended to toxify the sterile serum since severe shocks were obtained, and in one instance the 120-hour test proved acutely fatal. In another series, where the incubated serum was tested at intervals up to 56 hours, only moderate effects were obtained.

In the belief that incubation at 45 C. would assist the reaction, an experiment was tried in which pairs of tests were made at 1, 2, 3, and 6 hours, but the result was nil. It may be concluded, therefore, that labile rat serum does not readily become toxic in 1 c.c. dose when incubated at 37 or at 45 C.; on prolonged incubation, with consequent formation of sterile precipitate, it may show evidence of toxicity. Reference should be made to Table 142, Exper. B, in which a normal serum, kept at 45 C., was tested in 2 c.c. dose. It will be seen that some effect was obtained after incubation for 45 min. It would be interesting to

test the effect of incubation on the sensitized rat serum, since this seems to be considerably more labile than the normal. A short series of this kind, with tests up to 45 minutes, will be found under Exper. A, Table 137; the results were nil.

In regard to the second query, as to what happens when normal rat serum is incubated with egg-white, the experiments given in Table 136 may be offered by way of an answer. Since the addition of agar, peptone, trypanosomes, etc., readily induces production of anaphylatoxin, it might be expected that egg-white would effect a similar reaction. The seemingly positive results, however, must be accepted with reserve, since the test dose contained 4 c.c. of serum, which amount, by itself, may be toxic, as has been shown to be the case in Table 121. The failure to make a parallel series of tests with the same pooled serum, without any addition, practically invalidates the experiment.

TABLE 136
ACTION OF EGG-WHITE ON NORMAL RAT SERUM, RATIO, 1:5

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time at 37° C.	
A	1	207	4.8	45"	Very slight
	2	201	"	4' 5"	Slight
	3	202	2.4	15'	Practically nil
	4	205	"	30'	"
B	5	200	4.8	1'	Slight
	6	202	"	4'	Very slight
	7	194	"	15'	3'30"
	8	182	"	30'	Practically nil
	9	183	"	45'	3'40"
	10	204	"	60'	4'

The 2 experiments given in Table 136 were each made on the same day as the corresponding experiments of Table 135, and were intended as controls. As in those experiments, the egg-white and the pooled serum were warmed up separately to 37° C., and then mixed in the same ratio, 1:5. Mixture A consisted of 2.4 c.c. of egg-white and 12 c.c. of serum; while B was made up of 4.9 c.c. of the former and 24.5 c.c. of the latter. The 2 mixtures were made 3 weeks apart. After mixing, tests were made at once and at the intervals given in the table.

Egg-white is probably not as poisonous as peptone when injected intravenously, and if so, it can hardly be expected to induce toxicity, *in vitro*, unless it is used in relatively large amounts. In order to

toxify 2 c.c. of rat serum with peptone, it was necessary to use 25 mg. of the latter (Table 85); the amount of egg-white employed in Table 136 was but 4 mg. per 2 c.c. of serum. It will, therefore, be desirable to repeat these experiments with an increased quantity of egg-white.

Action of Distilled Water on Sensitized Rat Serum (Egg-White).

—Previous tests have shown that a sensitized rat shows little or no effect upon a second injection of antigen. Unlike the guinea-pig, it does not respond with an anaphylactic shock, certainly not with a fatal one. It has also been shown that the injection of dilute antigen may

TABLE 137
ACTION OF DISTILLED WATER ON SERUM OF RAT SENSITIZED TO EGG-WHITE

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time at 37 C.	
A	1	201	1	7'	Nil
	2	200	"	15'	"
	3	184	"	30'	"
	4	206	"	45'	"
B 3 + 1	5	201	4	35"	Very slight
	6	198	"	5'	Moderate
	7	206	"	15'	Fair
	8	199	"	32'	3'45"
C 6 + 1	9	206	7	1'	Very excited
	10	195	"	5'	2'20"
	11	196	3.5	20'	Very severe
	12	194	"	31'	3'25"
D 9 + 1	13	198	10	5'	2'10"
	14	197	"	15'	1'30"

The actual dose of serum injected was 1 c.c., except in Nos. 11 and 12, where it was 0.5 c.c.

produce a fatal shock; moreover, that a like amount of distilled water is equally fatal to a sensitized rat, but is without effect in a normal one. It is evident therefore, that the sensitized rat differs from the normal in its behavior to distilled water, in the same way that a sensitized guinea-pig differs from a normal one, as regards antigen. The sensitized rat injected with antigen, or dilute antigen, or distilled water, responds with an in vivo production of poison, though the amount produced may be insufficient to kill. Inasmuch as it has just been shown that sensitized rat serum, when mixed with antigen, almost immediately develops the poisonous property, it was in order to inquire as to the effect of distilled water on sensitized serum.

The experiments given in Table 137 were made with that object in view. For Experiments A, B, and C, 4 rats which had been sensitized by intravenous injection of 1 c.c. of egg-white, 16 days before, were bled; the bloods were defibrinated, centrifugated and the resulting sera were pooled (11 c.c.). The pooled serum, previously warmed to 37 C., was divided into 3 portions; to portions B and C, distilled water, likewise at 37 C., was added in amounts indicated below. Portion A served as a control without addition of water.

- A. 4 c.c. of pooled serum.
- B. 12 c.c. distilled water + 4 c.c. pooled serum. (3 + 1).
- C. 18 c.c. " " + 3 " " " . (6 + 1).
- D. 18 c.c. " " + 2 " " " . (9 + 1).

Mixture D was made on the following day in order to supplement the first 3 experiments; the necessary serum was obtained from a rat which was sensitized at the same time and in the same way as those used for the other tests.

The mixtures were at once placed at 37 C., B and C being tested immediately; the other tests were made at intervals, as indicated in the table. The dose injected represents only 1 c.c. of serum, except in tests Nos. 11 and 12, where it is 0.5 c.c. The effects produced cannot be ascribed to either constituent taken by itself, but are directly due to the action of the distilled water on the sensitized serum.

The control set A, with the undiluted serum, showed no effect even after incubation for 45 minutes. In Series B, with the least amount of water, no fatal shock was obtained until after incubation for 32 minutes (No. 8). On the other hand, in Series C, with twice the amount of water, rapid toxification occurred since the mixture killed when tested after 5 minutes (No. 10). This mixture also killed in 0.5 c.c. serum equivalent (No. 12). In Series D, unfortunately no immediate test was made, but the indication is that a severe, if not fatal, shock would have been obtained had a test been made about one minute after mixing; it is also probable that this mixture would have killed in dose of 0.5 c.c. serum equivalent. ,

The results here given should be compared with those in Table 144, where distilled water acted on the serum of rat sensitized to horse serum. It will be seen that the 9 + 1 mixture in that instance killed in 0.5 and even 0.25 c.c. serum equivalent (Nos. 22 and 23).

The symptoms following the injection of these mixtures were typical of anaphylatoxin, especially when the volume injected was

small, as in Nos. 8, 11, and 12; with the larger volumes, the shock was rapid and more of the quiet type. The time of death is figured from the end of the injection. The time of injection varied with the dose, being 10 to 20 seconds for 4 c.c.; 45 seconds for 7 c.c. and 1 minute for 10 c.c. The autopsies made 3 minutes after death showed the usual typical findings, though in Nos. 10, 13, and 14, where large doses were injected, a slight clot could be detected in the heart. This was entirely absent in Nos. 8 and 12. The presence of an extremely slight clot in No. 10, and its absence in No. 12, which received one-half the dose of the former, parallels the behavior of large and small doses of rabbit serum as regards production of clot (Tables 103 and 104).

These experiments demonstrate that a sensitized rat serum can be toxified by the addition of distilled water, and incubation. Furthermore, the toxicity thus developed is greater than that obtained by the action of undiluted antigen (Table 135). This fact as well as the great speed of poison-production is remarkable. The speed of reaction increases with the degree of dilution; in this respect, it corresponds to the observations made in connection with the spirillar paradox (page 782), the Neisser-Wechsberg phenomenon. A nonspecific reagent, distilled water, induces a more poisonous state, *in vitro*, as well as *in vivo*, than does specific antigen, in this case, egg-white.

The adsorption theories regarding anaphylatoxin production were materially weakened by the demonstration that a clear solution of peptone could toxify rat serum (Table 85). The evidence here presented of the action of distilled water may be said to effectually dispose of such theories, and since the reaction in this case involves a sensitized serum, it will be apparent that it has a direct bearing upon the phenomenon of anaphylaxis.

Action of Distilled Water on Normal Rat Serum.—It has been shown that distilled water exerts a remarkable action on sensitized rat serum; such being the case, it was in order to inquire as to the behavior of this reagent with normal serum. The first experiments made with this object in view are given in Table 138. For convenience in comparison, the experiments there given are designated as C and D, the dilutions corresponding to those of the same letters in Table 137.

Experiment D was made on the same day, as a control for the corresponding tests with sensitized serum of like dilution (Table 137, D); a comparison of these 2 experiments will show a striking difference. Thus, while the sensitized serum in the given dilution ($9 \div 1$) is

promptly toxified, within 5 minutes, the normal serum, under identical conditions as to dilution ($18 + 2$), temperature, and time, showed practically no effect. In view of the results obtained in Exper. C, it is reasonable to expect that a longer incubation of Mixture D would have resulted in poison-production.

For Exper. C, which was made 5 days later, a mixture of 36 c.c. of distilled water and 6 c.c. of fresh normal rat serum was made, each constituent being previously warmed to 37 C. A portion was tested at once (No. 1), while the balance was returned to the water bath to be retested at intervals, as indicated in the table. These tests serve as controls for those of Exper. C, Table 137, and the results deserve careful comparison. If the speed of the reaction was slow in Exper. D, it was equally so with this dilution of normal serum. In contrast with Exper. C of Table 137, where the mixture was toxified in 5 minutes, in this series the mixture failed to kill until after incubation for 45 minutes; not only the speed, but also the amount of poison produced was less, for while the 0.5 c.c. serum equivalent, in the former, was rendered toxic within 31 minutes, in the latter, the same result was reached only after incubation for 1 hour, 45 minutes. This experiment has been repeated a number of times, and it has been found possible to have a more reactive normal serum which will be toxified within 30 minutes. The individual resistance of the animals must be considered, and a better conclusion may be derived regarding the speed of reaction, if the inoculations are made in pairs, rather than as single tests. It will be shown in Table 141 that similar dilutions, kept at 45 C., may be toxified in 7 minutes.

It is desirable to state explicitly that the symptoms and findings in the fatal shocks (Nos. 4 and 8) were absolutely typical and indistinguishable from those caused by anaphylatoxin, or in specific anaphylaxis; severe dyspnea, spasms, violent convulsions, with autopsy showing permanent maximal distention of lungs, heart beating vigorously, with entire absence of clot is an ensemble that is thoroughly characteristic. The absence of clot is noteworthy, since in test No. 10 of Table 137, a very minute clot was found; this again indicates that the sensitized serum is more labile and responds with a more marked disturbance than does normal serum; the tendency toward early clotting does not mean that an excess of anaphylatoxin is present, but rather that the disturbance induced results in the simultaneous production of something (commonly called fibrin-ferment) which accelerates the coagulation change.

It will be seen from the foregoing that it is possible to produce a kill with normal rat serum by merely incubating it with distilled water. The actual amount of serum present in the fatal shocks (Nos. 4 and 8) was 1 and 0.5 c.c., respectively. It has been pointed out on page 805, that a normal rat serum, without any addition, may be rendered more or less toxic by prolonged incubation. The rate of reaction in such experiment is very slow; it is accelerated by the addition of distilled water, and is still more intensified when the serum is in the sensitized state.

The fact that normal rat serum, when diluted with distilled water and incubated, gave rise to anaphylatoxin was of such importance that it was desirable to examine more closely into the conditions involved.

TABLE 138
ACTION OF DISTILLED WATER ON NORMAL RAT SERUM AT 37 C.

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time at 37 C.	
C 6 + 1	1	209	7	40"	Nil
	2	211	"	15'	Very slight
	3	206	"	30'	Severe
	4	200	"	45'	3'10"
	5	210	3.5	60'	Moderate
	6	208	"	1 hr. 15'	Good
	7	201	"	" 30'	Severe
	8	209	"	" 45'	5'55"
D 9 + 1	9	208	10	5'	Very slight
	10	195	"	15'	" "

The dose represents 1 c.c. of serum, except in Nos. 5 to 8, where it is 0.5 c.c. The time of injection was 10 seconds for 3.5 c.c.; about 40 seconds for 7 c.c.; and 1 minute for the 10 c.c. dose.

It had been previously shown, in connection with Table 44, that while a mixture of agar and rabbit serum may be apparently toxified at 50 C., in reality, the poison was probably formed before the temperature had reached this level. A similar condition was pointed out apropos of Table 116, where marked primary toxicity was developed in rabbit blood, defibrinated and kept at 55 or 60 C. In view of these observations, it was in order to test the action of distilled water on rat serum at higher temperatures.

Two experiments made with this object in mind are given in Table 139. For these tests, and those given in Table 140, 20 rats were bled, yielding 50 c.c. of pooled serum, which was kept in cracked ice. Experiments A and B were begun 5 and 9 hours, respectively, after starting

to bleed. For the former, 72 c.c. of distilled water, at room temperature, were added to 12 c.c. of the serum (0 C.), ratio, 6:1; this mixture was tested at once, and the balance was placed at 55 C. and tested at intervals of 15 minutes.

In order to insure definiteness in results, the tests were made in pairs. It will be noted that 3 acutely fatal shocks and 2 near-kills were

TABLE 139
ACTION OF DISTILLED WATER ON NORMAL RAT SERUM (6:1) AT 55 C.

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time* at 55 C. (hr.)	
A	1	189	7	—	Nil
	1a	193	"	—	"
	2	190	"	$\frac{1}{4}$	Very near-kill
	2a	192	"	"	1'32"
	5	190	"	$\frac{1}{2}$	Slight
	3a	190	"	"	"
	4	194	"	$\frac{3}{4}$	Near-kill
	4a	193	"	"	Very slight
	5	197	"	1	Slight
	5a	198	"	"	3'40"
B	6	205	"	$1\frac{1}{4}$	3'28"
	6a	207	"	"	Very slight
	7	194	"	$\frac{1}{4}$	Slight
	7a	200	"	"	Very slight
	8	205	"	$\frac{1}{2}$	" "
	8a	208	"	"	" "
C	9	197	"	$\frac{3}{4}$	" "
	9a	197	"	"	" "
	10	182	"	$\frac{1}{4}$	" "
	10a	185	"	"	4'30"
	11	185	"	$\frac{1}{2}$	5'35"
	11a	185	"	"	Very slight
	12	205	"	$\frac{3}{4}$	" "
	12a	197	"	"	" "

* The injection time ranged from 17 to 43 seconds.

obtained out of 12 tests. In view of the early results in Nos. 2 and 2a, it is very certain that the full amount of the poison was produced within the first 15 minutes of the experiment. The irregularities in Series A, taken as a whole, as well as in the several pairs, afford a further excellent illustration of the variation in the resistance of guinea-pigs, rather than a change or decrease in the amount of poison.

It might be assumed from this experiment that distilled water does produce some poison in normal rat serum, even at 55 C., but such interpretation is not correct. It requires some time for a cold mixture, such as that employed, to attain a temperature of 55 C. An actual test with a like mixture of 72 c.c. of water, at room temperature, and 12 c.c. of water, at 0 C., showed that it took 6 minutes to reach 50 degrees; after that the rise was much slower, since in 11 minutes it had only reached 53 C. It is evident, therefore, that in the experiment with serum there was abundant time, in the 5 minutes needed to reach 50 C., to develop some poison. The fact that there was no increase after 15 minutes indicated clearly that poison-production did not go on at 55 C.

To prove that this explanation was correct, Exper. B was made. In this, the same pooled serum was used as in A. The distilled water (36 c.c.) and the serum (6 c.c.) were warmed separately at 55 C. for 5 minutes, then mixed and the mixture at once returned to the water bath. The tests, made at intervals of 15 minutes, gave practically negative results, thus demonstrating that no poison was produced at 55 C.

In order to meet the possible objection that the serum used in Exper. B had been changed by being iced for a longer time than that of the former experiment, it was decided to repeat Exper. A. For this purpose, 36 c.c. of distilled water, at room temperature, were mixed with 6 c.c. of the same pooled serum, at 0 C., this mixture was then placed at 55 C., and tested with the results given in Exper. C. The time from the start of bleeding to the beginning of this experiment was $10\frac{3}{4}$ hours. It will be seen that in this check trial, 2 of 6 tests proved fatal which indicates essentially the same amount of poison-production as in Exper. A. Consequently, the foregoing conclusion must be correct.

The important fact ascertained from the preceding experiments in Table 139 was that an exposure of rat serum at 55 C. for 5 minutes was sufficient to inactivate it as to poison-production by means of distilled water. This being the case, it was next in order to make similar experiments at 50 C. Fortunately, it was possible to use for these new experiments the same pooled serum which had been employed for those in Table 139. This serum had been kept in cracked ice, over night, and for Exper. A of Table 140, it was used 23 hours after the start of bleeding.

The procedure for Exper. A was the same as for the corresponding one of the day before (Table 139). The mixture was prepared by

bringing together 48 c.c. of distilled water, at room temperature, and 8 c.c. of pooled serum, at 0 C., after which it was placed at once at 50 C. The tests were made, in pairs, at intervals of 15 minutes, with results given in Table 140. The poison-producing capacity of the serum had not been injured by icing for about 23 hours. Of 8 tests, 6 proved fatal. The second pair of tests gave only slight or moderate effects, and if the experiment had been stopped at this point, it might have been supposed that the poison had decreased in amount; that such was not the case is

TABLE 140
ACTION OF DISTILLED WATER ON NORMAL RAT SERUM (6:1) AT 50 C.

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time* at 50 C. (hr.)	
A	1	207	7	$\frac{1}{4}$	3'40"
	1a	207	"	"	4'40"
	2	195	"	$\frac{1}{2}$	Slight
	2a	191	"	"	Moderate
	3	195	"	$\frac{3}{4}$	6'
	3a	195	"	"	6'10"
B	4	194	"	1	2'50"
	4a	194	"	"	3'
	5	195	"	$\frac{1}{4}$	1'30"
	5a	195	"	"	Nil
	6	203	"	$\frac{1}{2}$	"
	6a	203	"	"	Very slight
C	7	199	"	$\frac{3}{4}$	" "
	7a	199	"	"	" "
	8	185	"	$\frac{1}{4}$	3'22"
	8a	190	"	"	8' 2"
	9	186	"	$\frac{1}{2}$	3'
	9a	189	"	"	2'50"
	10	190	"	$\frac{3}{4}$	Severe
	10a	187	"	"	2'35"

* The injection time ranged from 25 to 43 seconds.

seen from the next 4 tests which proved fatal. This merely again illustrates how the variable resistance of the guinea-pig may lead to wrong conclusions.

The excellent results in Exper. A of this series, if taken by themselves, could easily be interpreted as proving that anaphylatoxin was made by incubation at 50 C. This conclusion, however, might be fallacious for reasons previously cited, so to test this point, Exper. B was

made. For this, 36 c.c. of distilled water and 6 c.c. of the same pooled serum (24 hours) were warmed separately at 50 C. for 17 minutes; after which they were mixed, and the flask was returned at once to the water bath. Tests were now made at intervals of 15 minutes, with the rather surprising result shown in Table 140, B. The effects were practically nil, with the exception of test No. 5, in which death occurred as rapidly as in No. 2a of Table 139.

The marked absence of toxicity could not be explained by any change in the serum, since this experiment was made an hour after Exper. A. The only explanation seemed to be that exposure of the serum to 50 C. for 17 minutes rendered it inactive. However, as a control, Exper. C was made. Here again, the same pooled serum was used, it now being 26 hours from the start of bleeding; for the last 2 hours, the serum had been kept at room temperature. A mixture of 36 c.c. of distilled water and 6 c.c. of the serum, both at room temperature, was made, and at once placed at 50 C. When tested, as before, in pairs, it showed that the serum was just as reactive as when used for Exper. A. Consequently, the failure of Exper. B was due to a change in the matrix of the poison, caused by exposure for 17 minutes to a temperature of 50 C. Further, it is evident that the poison-production in A and C occurred probably while the temperature was rising to that level, and not after it had been reached.

The fact that the preliminary heating of normal rat serum, at 50 C. for 17 minutes, altered it so that when mixed with distilled water and incubated at 50 C. practically no poison was produced, naturally led to a similar experiment at 45 C. For this purpose, 8 rats were bled and the resulting 30 c.c. of pooled, clear serum were at once placed in cracked ice. In Exper. A, the serum was used 5 hours after the start of bleeding. The mixture was made by bringing together 36 c.c. of distilled water, at room temperature, and 6 c.c. of the serum, at 0 C.; it was at once placed at 45 C., and tests were made, in pairs, at intervals of 15 minutes. It will be seen from the results given in Table 141, that every test gave a fatal shock, thereby indicating perhaps that more poison had been produced than in the corresponding experiments at 50 and 55 C. (Tables 140, 139).

Although unlikely, it was decided to ascertain whether exposure of the serum at 45 C. would impair its capacity to produce poison. For this purpose, 36 c.c. of distilled water and 6 c.c. of pooled serum were warmed separately at 45 C. for 35 minutes, after which they were

mixed and returned to the water bath. The results of these tests are given in Exper. B (Table 141); it will be seen that such treatment of the serum does not alter its reactivity. In other words, the matrix of the poison is not injured by heating at 45 C. for 35 minutes, whereas at 50 C. for 17 minutes, or at 55 C. for 5 minutes, actually renders it nonreactive to distilled water.

A striking fact brought out in connection with the experiments given in Tables 139, 140, and 141, was the possibility to toxify the

TABLE 141
ACTION OF DISTILLED WATER ON NORMAL RAT SERUM (6:1) AT 45 C.

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time* at 45 C.	
A	1	180	7	¼ hr.	3'58"
	1a	184	"	"	3'20"
	2	190	"	½ hr.	1'50"
	2a	196	"	"	3'15"
	3	185	"	¾ hr.	4' 5"
	3a	190	"	"	2'55"
B	4	204	"	¼ hr.	3'10"
	4a	204	"	"	2'13"
	5	190	"	½ hr.	Very severe
	5a	190	"	"	2'20"
	6	178	"	¾ hr.	3'
	6a	172	"	"	3' 5"
C	7	200	"	7 min.	2'25"
	7a	194	"	"	2'50"
	8	200	"	12 min.	Slight
	8a	196	"	"	Very slight
	9	198	"	17 min.	3'45"
	9a	195	"	"	3'15"

* The injection time ranged from 15 to 40 seconds.

serum in the 6:1 mixture within 15 minutes. In Table 138, this result was apparently not reached until after incubation at 37 C. for 45 minutes. This fact indicated that a temperature higher than 37 C. was more favorable to poison-production, and, if so, it was reasonable to expect that at 45 C. a toxic dose could be obtained at maximal speed.

Accordingly, Exper. C was made to test this point. For this, the same pooled serum was employed as in Experiments A and B. The distilled water (36 c.c.) and the serum (6 c.c.) were warmed separately

at 45 C. for 15 minutes, and then combined, the mixture being at once returned to the water bath. The tests were planned for 5, 10, and 15 minutes, but the actual time from that of mixing until end of the injection was 7, 12, and 16 minutes. The mixture was toxified within 7 minutes, so that a dose, the serum equivalent of which was 1 c.c., was acutely fatal. Here again, as in Exper. A of Table 140, the second pair of tests failed to give anything more than mild effects, the guinea-pigs being unquestionably more resistant than the other 4. In another experiment, made with the same serum and at the same time as Exper. C, the conditions being alike, after incubation for 30 minutes, the mixture killed in a dose, the serum equivalent of which was 0.5 c.c.

TABLE 142

ACTION OF DISTILLED WATER ON NORMAL RAT SERUM (6:1), AT 45 C. (A); UNDILUTED SERUM AT 45 C. (B)

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time* at 45 C. (min.)	
A	1	193	7	7	Very slight
	1a	196	"	"	"
	2	193	"	11	3'
	2a	190	"	"	2'38"
	3	190	"	16	2'50"
	3a	190	"	"	2'30"
B	4	192	2	15	Slight
	5	193	"	30	"
	5a	193	"	"	"
	6	185	"	45	Very slight
	6a	185	"	"	Moderate

* The injection time for Nos. in Exper. A was 20 to 30 seconds; Exper. B, 3 to 8 seconds.

As the speed of toxification was of importance, it was decided to repeat Exper. C with another serum. Accordingly, on the following day, 6 rats were bled and yielded 21 c.c. of an opalescent, lipoidal serum. As before, 36 c.c. of distilled water and 6 c.c. of this serum were warmed separately at 45 C. for 15 minutes, then combined and the mixture returned to the water bath. It was tested at 7, 11, and 16 minutes, this time being that from moment of mixing till the end of the respective injections. The results of this experiment are given in Table 142, Exper. A, and are to be compared with those of Exper. C, in the preceding table.

While the first 2 tests were practically negative, those at 11 and 16 minutes were acutely fatal. The autopsies of Nos. 2 and 2a made, respectively $5\frac{1}{2}$ and 4 minutes after death, showed the presence of very slight clot in each heart. This may be taken to mean that half the dose used would have killed without tendency to clot, as has been shown to be the case in Exper. C., Table 137. In Nos. 3 and 3a, the examination at $4\frac{1}{2}$ and 3 minutes after death revealed no sign of clot, the heart beating vigorously; in all 4, the lungs were in maximal distention. The failure to toxify within 7 minutes may have been due to the fact that the serum was lipoidal; it is also possible that the guinea-pigs happened to be resistant, in the same way as Nos. 8 and 8a of Table 141.

Inasmuch as it was possible that the excellent results obtained in these speed experiments could be due to the direct action of the heat of 45 C., it was deemed best to determine this point by suitable controls. For this purpose, 6 c.c. of the same pooled serum were placed at 45 C., and after incubation for one-fourth, one-half, and three-quarters of an hour, portions of 1 c.c. were tested, 3 pairs of tests being thus made. The effect in all 6 tests was nil.

As a further, and more conclusive, test, 10 c.c. of the same pooled serum were incubated at 45 C. and then tested in 2 c.c. dose, at the same intervals. The results of this series are given in Exper. B, Table 142. Even 2 c.c. of undiluted serum is not toxified at 45 C., in the time employed. No. 6a, however, showed a moderate reaction with some dyspnea, spasms, and was nearly thrown; this result may be interpreted as due to a very susceptible animal, but it is also possible that the serum was beginning to show some evidence of poison-production, as it was at this time incubated at 45 C. for 45 minutes. This experiment is deserving of repetition and extension.

It is indeed possible that the normal rat serum when kept at 45 C. for some time may become labilized, so that on subsequent dilution with distilled water (6:1), at 45 C., poison may be produced almost instantaneously. With this in mind, some of the same pooled serum as used in Experiments A and B, Table 142, was placed at 45 C.; after incubating for one-fourth, one-half, and three-fourths of an hour, portions of 1 c.c. were removed and each was diluted with 6 c.c. of distilled water, also at 45 C., and the resulting mixtures were at once injected. The actual contact of the heated serum with the distilled water, from the time of mixing until the end of injection, was but 30 to 45 seconds.

The tests which were made in pairs, at one-fourth and one-half of an hour, showed practically no effect. Of the tests made at the three-quarter hour interval, 1 proved fatal in 19 minutes, while its companion showed only slight effects. This experiment is extremely suggestive of the possibility of producing a nonspecific sensitized, or labilized serum by mere heat action.

Since it has been shown that the mere heating of normal rat serum at 50 C. induces an alteration which is recognized by the fact that such heated serum is no longer toxified by distilled water, it was of interest to see if this could be rendered toxic by means of agar. The experiments made with this objective showed that agar-gel could toxify a rat serum which had been heated at 45 and 50 C. for half an hour. The results of these trials are given in Table 55, where it will be seen that the same serum which was heated to 50 C. for half an hour was less reactive with agar than when heated at 45 C. for the same time. The former on agar treatment was not toxified in dose of 1 c.c., but with 2 c.c. it was possible to get some kills. Since distilled water is unable to toxify normal rat serum which has been heated at 50 C. (Table 140), or at 55 C. (Table 139), it follows that the suspensoid agar is considerably more active as an inducer of the reaction. This, of course, can be expected when it is recalled that agar can toxify a rat serum in ratio of 1:400,000.

It may be assumed that exposure to the temperatures mentioned inaugurates a change in the matrix which, if continued, results in a permanent modification which is either irreversible, or such that it cannot undergo the intramolecular change necessary to poison-production. A moderate exposure may partially change the matrix-complex, so that while water is unable to cause reversion, or transformation, this can be done by the more powerful agar. In other words, the labile matrix is subject to 2 types of change: First, it undergoes aggregation at a relatively low temperature, lower than that which is effective with the other serum-proteins, forming a tautomeric, inactive modification; and second, it is transformed readily into another tautomeric, physiologically active form, anaphylatoxin.

Since agar was able to toxify normal rat serum which had been heated to 50 C. for half an hour, it was desirable to see if reversion changes occurred when such heated serum was kept at 0 C. for some time. If prolonged cold exerted an action of this kind, then subsequent treatment with distilled water should give evidence of poison-production. An experiment having this purpose was accordingly made.

Nine c.c. of a fresh pooled serum were divided into 2 portions: One of these (A), 7 c.c., was placed at 50 C. for half an hour, then in cracked ice and set aside in an ice-box for 22 hours; the other portion (B), 2 c.c., was iced directly for the same time. The next day, both portions were diluted with 6 parts of distilled water and placed at 45 C. After incubation for 15 minutes, the control, or portion B, produced acute fatal shock in 2 guinea-pigs, the dose being 1 c.c. serum equivalent. By contrast, the mixture with the previously inactivated serum, portion A, likewise tested in pairs, after incubation for one-fourth, one-half, and three-fourths of an hour gave practically no effect, the dose being the same as for the controls. It is evident, therefore, that prolonged keeping at 0 C. does not cause a reversion, or restore the lability to a serum which has been inactivated by heating to 50 C. for half an hour.

To explain the results here presented from the ferment standpoint, it would be necessary to assume that the ferment is either destroyed or that an antiferment is formed when the serum is heated for a few minutes at 50 C. This view is difficult to reconcile with the mass of facts accumulated in this investigation. It seems to be more reasonable to assume that the matrix is an extremely labile body which readily undergoes intramolecular changes yielding either an inert, or a toxic modification.

Action of Horse Serum on That of Rat Sensitized to Horse Serum.

—There is reason to believe, from the results presented in Table 133, that egg-white, which is totally alien to the blood stream, induces a greater change in the physical state of the plasma constituents, that is, labilizes more energetically, than any normal serum. This would seem to be, a priori, what might be expected, and the following in-vitro experiments actually appear to indicate less lability in the serum of rats sensitized with horse serum than in that with egg-white (Table 135). This, however, holds true only when the antigen is used; with distilled water, the sensitized sera behave about alike (Tables 137 and 144).

The rats employed for the following experiments were sensitized by 3 intravenous injections, 0.01, 0.5, and 3 c.c. of horse serum, respectively; the injections were given in the course of 2 months. Of these, 2 were bled 17 days after the last injection, and the pooled serum (7 c.c.) was used for Exper. A. On the 18th day, 3 more were bled, yielding 6 c.c. of pooled serum which was employed for Exper. B.; for Exper. C, 3 others were bled, also on the 18th day, and this gave

8 c.c. of pooled serum. The pools were warmed in a beaker of water at about 37 C., as also was the antigen, before making the mixtures. These were prepared according to the subjoined plan.

- A. 1.4 c.c. horse serum + 7 c.c. sensitized rat serum (1:5).
 B. 1.2 c.c. " " + 6 c.c. " " " (1:5).
 C. 1.6 c.c. " " + 8 c.c. " " " (1:5).
 D. 1.4 c.c. " " + 7 c.c. normal " " (1:5).

The mixtures were kept in the warm water, and the tests were made at the times indicated in Table 143. The time given is that from the moment of mixing until the end of injection. The dose given represents 2 c.c. of sensitized serum, except in Nos. 7 and 8, where it is 3 c.c.

TABLE 143

ACTION OF HORSE SERUM ON SENSITIZED RAT SERUM (1:5), A, B, C, E; CONTROLS D AND F

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time at 37 C. (min.)	
A	1	205	2.4	1	Practically nil
	2	187	"	3	Near-kill
	3	188	"	15	Very severe
B	4	195	"	2	Intense quiet shock
	5	187	"	5	5 hrs. " "
	6	194	"	8	12 hrs. " "
C	7	177	3.6	1'15"	2¼ hrs. " "
	8	195	"	4	10 hrs.
D	9	196	2.4	1	Nil
	10	199	"	3	"
	11	189	"	5	"
E	12	185	4.8	1'20"	4'20"
	13	175	2.4	7	Slight
	14	184	"	15	Very slight
	15	178	"	30	"
F	16	181	4.8	3'30"	4¼ hrs. Quiet shock. Temp. in 4 hrs., 23.8 C.

In Exper. A, very severe effects followed the injection of Nos. 2 and 3. Without doubt, a dose of 4.8 c.c., such as used later (Table 135) would have killed, but the mixture was certainly not as toxic as in that experiment where 1.4 c.c. was fatal. The symptoms were those of typical anaphylactic shock: dyspnea, spasms, convulsions and the animals were thrown. Prompt recovery followed. The effect of the first injection, which was made within 1 minute, was nil.

On the other hand, the tests in Experiments B and C, although made under nearly identical conditions, presented an entirely different pic-

ture. With the exception of No. 8, there was an entire absence of violent effects, the animals reacting with an intense quiet shock; they showed prostration, became paralyzed, and the temperature dropped quickly, that of No. 5 in 4 hours being 25.4 C. Death occurred after some hours; at autopsy, the blood was found to be free from clot, a condition usually encountered where gradual asphyxiation takes place. The difference in the results of Exper. A, as contrasted with those of Expers. B and C, was probably due to variations in the temperature at which the mixtures were kept, and for that reason, in all subsequent experiments of this kind (Table 135, etc.), the Roux water bath was employed.

Experiment D served as a control and showed that the addition of horse serum to normal rat serum, under the conditions given, does not produce any poisonous effect. It would appear from the preceding experiments that the blood of rats sensitized to horse serum is not as reactive as that from such as have been treated with egg-white (Table 135). To fully establish this point, however, it was necessary to make further tests under more exact conditions, as to temperature and dosage.

For this purpose, 5 rats were sensitized by intravenous injection of 0.25 c.c. of horse serum; they were bled on the 19th day, yielding 14 c.c. of pooled serum. This, as well as some antigen, and distilled water were first warmed in a Roux water bath at 37 C., and thereupon 2 mixtures were made: 2 c.c. of horse serum + 10 c.c. of sensitized rat serum (1:5), and 0.8 c.c. of distilled water + 4 c.c. of sensitized rat serum (1:5). These were returned to the water bath and tested at times indicated in Table 143.

The control Exper. F resulted in a quiet shock, with paralysis, and drop in temperature to 23.8 C., at half an hour before death; the effects being much the same as those in Expers. B and C, including a non-coagulable blood. This test shows that the serum pool possessed a primary toxicity in the dose employed, or, 4 c.c. It might be assumed that the slow toxic action observed was due to the action of the distilled water on the sensitized serum. In view of the action of water on normal rat serum, it may be expected to have an even more rapid action on one that is sensitized, and this is actually the case, but the dilutions must be considered (Table 144). The fact that a similar test with the labile egg-white serum (Table 135, No. 6) showed no effect after incubation for 4 minutes may be taken as evidence against the assumption.

Although test No. 16 must be interpreted as showing that the pooled serum possessed a primary toxicity in dose of 4 c.c., the result of test No. 12 in Exper. E cannot mean anything else than that an increase in toxicity had occurred as a consequence of the short contact with the antigen. The serum being on the threshold, as it were, it was easy to increase the poisonous property so that an acute death occurred. The failure to produce severe effects in Nos. 13 to 15, where half the dose was used, shows that the amount of poison produced was not very great. In the corresponding experiment with egg-white (Table 135, No. 3), 1.4 c.c. of the mixture killed; this fact again indicates that the serum sensitized by means of horse serum is not as reactive to antigen as that with egg-white.

Action of Distilled Water on Serum of Rat Sensitized to Horse Serum.—Having shown that the mixture of horse serum and sensitized serum gave rise to poison-production, in vitro, though perhaps less readily than in the case of egg-white, it was desirable to demonstrate next the action of distilled water on such sensitized serum. For this purpose, 4 rats which had been sensitized by intravenous injection of 1 c.c. of horse serum 15 days before, were bled, and the pooled serum (16 c.c.) and some distilled water were warmed to 37 C. in the Roux water bath, after which 4 mixtures were made.

- A. 4 c.c. of the pooled serum, with no addition, to serve as control
- B. 12 c.c. distilled water + 4 c.c. pooled serum. (3 + 1)
- C. 24 c.c. " " + 4 c.c. " " (6 + 1)
- D. 36 c.c. " " + 4 c.c. " " (9 + 1)

These mixtures were then placed at 37 C., and tested at intervals, as indicated in Table 144.

A study of Table 144 in connection with the corresponding one with egg-white (Table 137) is of interest. It justifies the conclusion that rat serum which is sensitized to horse serum behaves with distilled water in the same way as that sensitized with egg-white. It will be seen (Exper. A) that in both sets the undiluted serum, in dose of 1 c.c., was practically without effect. Dilution B, in dose of 1 c.c. serum equivalent produced a severe shock after incubation for 30 minutes, while the corresponding test in Table 137, No. 8, gave an acute kill. This difference does not necessarily mean that the latter mixture contained more poison, since it is possible that the factor of individual variation in guinea-pigs played a part.

On the other hand, a comparison of the C dilutions might favor that of Table 144, were it not for this variable factor of animal resistance. It is to be noted that the mixture was toxic after incubation for 5 minutes, in dose of 1 c.c. serum equivalent; and likewise, toxic in half that dose, after 15 minutes. The 0.25 c.c. serum equivalent produced very severe shocks in 4 of 6 tests.

The tests with Dilution D likewise showed rapid production of poison, and it is to be specially noted that the dose of 0.25 c.c. serum

TABLE 144
ACTION OF DISTILLED WATER ON SERUM OF RAT SENSITIZED TO HORSE SERUM

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time at 37 C. (min.)	
A	1	180	1	5	Nil
	2	200	"	15	Very slight
	3	197	"	30	Practically nil
	4	203	"	45	Moderate
B 3 + 1	5	205	4	1/2	Nil
	6	204	"	5	Slight
	7	196	"	15	Very slight
	8	182	"	30	Severe
C 6 + 1	9	206	7	1	Very slight
	10	203	"	5	3/50"
	11	192	3.5	15	4/53"
	12	185	1.75	30	Severe shock
	13	181	"	45	Slight
	14	183	"	60	Moderate
	15	180	"	75	Very severe
	16	198	"	90	Near-kill
	17	181	"	105	Severe
D 9 + 1	18	210	10	1/20"	Very slight
	19	207	"	5	2/40"
	20	200	5	15	Severe, quiet
	21	208	"	30	Very severe
	22	208	"	45	3'
	23	203	2.5	60	3/40"
	24	204	"	75	Moderate

The injection time in Exper. B was 10 to 15 seconds; the 7 c.c. dose was given in 17 to 40 seconds, while the 10 c.c., in about 1 minute. The autopsies made 5 minutes after death were perfectly typical in all but No. 19, where a very slight clot was found (bulk effect).

equivalent caused acute death at the 1-hour period (No. 23). These results should be compared with those in Table 138, which shows the behavior of normal serum under like conditions. A comparison may also be made with Tables 141 and 142, but the mixtures in the latter were incubated at 45 C., whereas those in this series (Table 144) were kept at 37 C.

This series of experiments confirms and extends the results obtained with the serum of rats sensitized to egg-white. There seems to be no

doubt but that the serum of a sensitized rat is considerably more labile than normal serum. It may be desirable, however, to make further tests with the latter, the mixtures being kept at 37 C., in order to make certain that the results given in Table 138 hold for other normal rat sera. The rapid toxification, even to 0.25 c.c. serum equivalent, by the mere addition of water is a fact of much importance. This, and the further fact that rat serum, on incubation with clear solution of peptone, etc., develops anaphylatoxin, indicates that a suspended solid is not necessary to this reaction. In other words, adsorption theories may find it difficult to account for the production of poison under these conditions.

TABLE 145

ACTION OF RABBIT, BEEF, AND GUINEA-PIG SERUM ON CORRESPONDING SENSITIZED RAT SERA (1:5)

Exper.	Guinea-Pig		Mixture		Result
	No.	Weight	Amount (c.c.)	Time at 37 C.	
A Rabbit	1	203	4	50"	Moderate
	2	197	"	5'20"	Slight
B Beef	3	195	4.8	50"	Slight
	4	187	"	5'	5'
	5	178	1.9	18'	3'15"
C Beef Control	6	203	4.8	35"	Moderate
	7	199	"	5'20"	Nil
	8	205	2.4	15'	Severe
D Guinea-Pig	9	178	4.8	4'20"	3'45"
	10	177	1.2	15'	Moderate

Action of Rabbit, Beef, and Guinea-Pig Serum on Corresponding Sensitized Rat Sera.—A limited number of tests were made with rat sera sensitized by means of other antigens than those hitherto mentioned.

Only one experiment (A) was made with the object of producing an in-vitro poison by combining rabbit serum with the correspondingly sensitized rat serum. Three rats which had received an intravenous injection of 0.25 c.c. of rabbit serum 16 days before were bled, yielding 7 c.c. of pooled serum. This and the antigen were warmed at 37 C., then combined in the ratio of 1:5; a test was made at once, the balance being returned to the bath and retested later. The results as shown in Table 145, A, were very moderate.

A test similar to the foregoing was made with beef serum. Four rats which had been sensitized by intravenous injection of 0.25 c.c. of

beef serum 16 days before, were bled, yielding 10 c.c. of pooled serum. This, together with some beef serum, was warmed at 37 C., then combined in the ratio of 1:5 and tested in the usual way. In view of the fact that beef serum itself may be very toxic, a control test, Exper. C, was made by adding beef serum to normal rat serum, both having been previously warmed to 37 C.

Lastly, Exper. D was made with guinea-pig serum as antigen. The rats were sensitized by intravenous injection of 0.25 c.c. of the serum. They were bled 24 days later, and the usual 1:5 mixture was made and tested with the result given in the table.

With regard to the rabbit serum test, Exper. A, it would appear that the serum was less active than that sensitized with horse serum. Further tests may be desirable to determine whether this difference is constant. By contrast, in Exper. B, the sensitized serum responded to its antigen, beef serum, quite promptly, since 2 deaths were obtained. The autopsies in Nos. 4, 5, and 9 were in every way typical. The severe shock in the control test No. 8 may indicate that the beef serum may have some toxifying action on normal rat serum. In Exper. D, with guinea-pig serum as antigen, a positive result was apparently obtained after incubation for 4 minutes. In the absence of a number of controls with the same pooled serum, caution must be used in accepting this as evidence of poison-production, since it has been shown in Table 121 that rat serum may be fatal in dose of 4 c.c.

NONCOAGULABILITY OF BLOOD IN SHOCKED GUINEA-PIGS

One of the effects noted in connection with anaphylactic shock is a more or less incoagulable blood. This condition is usually looked upon as a secondary phenomenon, whereas, in reality, it should be considered as a primary effect which proceeds, *pari passu*, with the production of poison; that is to say, noncoagulation and poisonous action are undoubtedly twin manifestations of one and the same reaction. It is not the intention, at this point, to consider the question of coagulation in general, but rather to emphasize the blood condition in shock, more especially in the guinea-pig.

It is a well known fact that dogs in anaphylactic shock yield an incoagulable blood which has been compared with that of peptone- or proteose-poisoning. On the other hand, in shock guinea-pigs, the results obtained hitherto have not been at all definite. Indeed, it is doubtful whether any one had obtained from this species a blood which

showed more than a moderate degree of retardation. Delayed coagulation is found in ordinary asphyxia; it is therefore to be expected in protracted anaphylactic death. It has been frequently observed, in the course of these studies, that in guinea-pigs which died an hour or more after anaphylatoxin injection, the examination made 20 to 30 minutes, or later, after death, showed the blood in the heart to be fluid and free of the slightest trace of clot. Instances of this kind, for example, were noted in connection with Table 143.

Even in the acute specific anaphylactic shock, where death occurs in from 3 to 4 minutes, the heart when examined 12 to 15 minutes later, and even as late as 24 minutes after death (Table 146, No. 2), has been found to be free of clot. When, however, some of the blood was drawn from the exposed heart with the aid of a syringe and transferred to a small test tube, it coagulated regularly in from 4 to 6 minutes, whereas normal blood, under like conditions, clotted in half this time. This result was obtained, no matter whether the blood was drawn 2 or 20 minutes after death. For a long time all attempts to produce in the shocked guinea-pig a strictly noncoagulable blood, corresponding to that of the dog, led to no other result than a mere delay in the process.

A blood may be rendered incoagulable by contact with the pleura; this is especially true in shocked animals. The following observation may serve to illustrate this point: A guinea-pig sensitized to egg-white was given a second injection of 2 c.c. 11 days later. Immediately after the injection, the heart was exposed and the blood was drawn up into a syringe previously rinsed in distilled water; it had been planned to remove a large amount of blood and transfer it as quickly as possible to the test tube, but the fall in blood pressure was so marked and so rapid that, although the heart was entered within $1\frac{1}{2}$ minutes after the end of the injection, it required nearly 3 minutes to draw up $2\frac{1}{2}$ c.c. of blood. When transferred to the test tube, this blood clotted firmly within 2 minutes, the total time from the start of injection being $6\frac{1}{2}$ minutes; within a minute after the blood had clotted in the tube, the heart was opened and found to be entirely free of any sign of coagulation. The opening of the heart allowed considerable blood to flow into the pleural cavity, and some of this was at once transferred by means of a syringe to a test tube. This blood remained fluid for more than 24 hours, whereas the portion which was taken direct from the heart clotted in 2 minutes.

It is well known that the circulating blood which has been rendered incoagulable by an injection, after a variable interval, returns to its previous normal state. Thus, the blood of a dog, after peptone injection, may be incoagulable within 2 minutes, and this condition may persist for an hour or more. The fact that in shocked guinea-pigs, the blood begins to clot within the heart in about 15 minutes after the injection indicates a similar return to the normal state. The poisonous property which develops in the blood in shock, likewise does not remain intact in the blood vessels, but rapidly recedes.

It seemed reasonable, therefore, to assume that the toxic and non-coagulable properties were essentially expressions of one phase of colloidal disturbance. The enormous mass of the body might be looked upon as exerting a reversive influence, which becomes evident by the disappearance of toxicity and return of coagulability. To demonstrate,

TABLE 146
NONCOAGULABILITY OF BLOOD IN ACUTE SHOCK IN THE GUINEA-PIG TREATED WITH EGG-WHITE

Guinea-Pig		Time* of Bleed	Result
No.	Weight		
1	195	1'45" - 2'45"	Solid clot in 2 min.
2	230	2'35" - 3'20"	No clot for 24 hr.
3	190	3' - 4'10"	" " " 1 hr. Then lost
4	178	3' 8" - 4' 3"	" " " 7 hr.; clotted in 19 hr.

* The time is that from the end of injection during which blood was drawn into the pipet.

therefore, the presence of a really incoagulable phase in the shocked guinea-pig, it was necessary to modify the technic so as to insure the removal of the blood from the reversive influence of the body-mass as quickly as possible.

It was also desirable to avoid all unnecessary contact with glass, water, metal and even air bubbles. Hence, the syringe, which had been used for much of the early work in transferring blood from the heart to the test-tube, was discarded, and replaced by the clean, dry heart pipet, from which the rod was removed. By applying a little suction, after the tip was inserted into the exposed heart, it was possible to obtain 2 or 3 c.c., at maximal speed. The drawing up of air bubbles was most carefully avoided. As soon as the desired amount of blood was drawn, the pipet was sealed with vaselin.

The procedure just given was followed in the tests given in Table 146. The guinea-pigs had been sensitized to egg-white, and the second

injection of 1 c.c. was given intravenously on the 13th day (Nos. 1 and 2), the 14th day (No. 3) and on the 81st day (No. 4). Shock symptoms at once became manifest. The section of Nos. 1 and 2 was begun at once after the injection, while that of Nos. 3 and 4 was delayed for 2 minutes. The time required to fully expose the heart was only 30 to 45 minutes.

It will be seen in Table 146 that the blood which was transferred within $2\frac{1}{2}$ minutes after the injection clotted as rapidly as the normal one. This means, of course, that there had not been sufficient time to induce the fullest possible dislocation within the plasma. On reference to Table 132, it will be seen that the shocked blood, at about this point, is likewise unchanged as to toxicity. On the other hand, blood drawn after $2\frac{1}{2}$ minutes had elapsed from the time of the injection showed perfect noncoagulability for hours. The blood of No. 3, in about 20 minutes showed a slight viscosity, but this disappeared in about 5 minutes, and the blood remained perfectly fluid, the last examination being made at the end of one hour. The accidental loss of the material prevented further observations. In No. 4, there was absolutely no sign of thickening or viscosity, and the fluid blood was under observation for 7 hours; the next morning, 19 hours after drawing, the contents of the tube had firmly clotted; coagulation, however, had not taken place until after the corpuscles had completely settled. The blood of No. 2 showed in 15 minutes a slight viscosity, but this soon disappeared, and the blood remained perfectly fluid for more than 24 hours, after which observations were discontinued.

These 3 positive tests conclusively show that it is possible to obtain noncoagulable blood from a shocked guinea-pig. It must be remembered that the bloods of different species, and even of the same species, may vary in their capacity to form anaphylatoxin, and also in their response as to alteration in coagulability. Differences of this kind have been touched on in Parts 5 and 7. It would be of interest to extend the series of tests given in Table 146, so as to ascertain the time at which coagulability is restored. Further, it is desirable that the same method be applied to other species in the shock state.

A number of tests were made with the noncoagulable blood of No. 2 to see if it was capable of clotting. On the supposition that a large glass surface favored clotting, a portion was drawn up into a capillary tube and freely moved back and forth, but there was no evidence of coagulation for the duration of the experiment, which was

30 minutes. As a check, it may be added that some blood was drawn up into a like capillary from the heart of No. 4, 20 minutes after the injection; this coagulated into a solid in 5 minutes. Consequently, it may be inferred that a large glass surface does not particularly aid clotting. For further tests, the noncoagulable blood of No. 2 was placed in portions of 0.5 c.c. in 4 small test tubes; the addition of one-fourth c.c. of distilled water to No. 1 caused it to clot in about 8 minutes; the addition of one-fourth c.c. of normal rat serum likewise effected coagulation in 7 minutes; the addition of one-fourth c.c. of a dilute CaCl_2 solution resulted in a clot in from 6 to 8 minutes; while the most rapid effect was obtained by adding a few drops of KCNS, which made a firm clot in 3 minutes.

The general conclusion to be drawn from this work is that the guinea-pig, when subjected to specific shock, responds with the production of a poisonous as well as noncoagulable state of the blood. The 2 results are temporary in character, and may be considered as a passing phase which is influenced by the mass action of the plasma, and especially that of the tissues. The timely removal from the body, thereby avoiding this mass action, stabilizes the condition so that incoagulability and toxicity persist and can be demonstrated.

SUMMARY

The rat has been found to be an invaluable reagent in the study of anaphylaxis. After previous sensitization, the test injections of corresponding antigens, such as egg-white, and diverse sera, yield, at most, a moderate respiratory disturbance. This refractory state concerning specific anaphylactic shock parallels the tolerance for multiple doses of anaphylatoxin, as well as to agar and peptone injections.

A more severe shock, and even death, follows the injection of very dilute antigen into sensitized rats, but is without effect in normal rats. The real agent in this shock is distilled water.

Nonspecific anaphylactic shock may be induced in sensitized rats by the injection of distilled water, in amounts which have no effect upon the normal rat. Salt solution in like dose can be tolerated with practically no effect.

The guinea-pig sensitized specifically is not more susceptible to distilled water than is the normal one. The relative behavior of the rat and guinea-pig to distilled water is paralleled in the relative ease with which the serum of the former, as compared with that of the latter,

is toxified by agar. Normal guinea-pigs on rapid injection of very large doses of saline, and especially of distilled water, respond with shock effects.

The blood and perhaps the tissues of the sensitized rat are in a more labile state than in the normal rat.

The blood of the guinea-pig, which has been subjected to anaphylactic shock, if withdrawn at the proper time, is strictly noncoagulable as well as toxic.

A poisonous state is demonstrable in the blood of shocked guinea-pigs by the transfusion method. Even 2 c.c. of such blood, transfused at the proper time, may cause acute fatal shock in the recipient.

The results are more marked in animals sensitized to the strictly alien egg-white than in those which had been treated with horse serum. With a reaction time of about 1 minute, the blood is nontoxic, but becomes so when this reaction time is about 3 minutes. A reversion tends to occur and is indicated by the rapid recovery in the nonfatal shock.

A like poisonous state is demonstrable in the blood of sensitized rats after injection of very dilute antigen. The rat sensitized to the alien egg-white yields more poison than that treated with horse serum. The transfused blood of a desensitized rat behaves like that of normal rats.

A mixture of egg-white and sensitized rat serum, at 37 C., is toxified in 4 minutes, and may even kill in dose of 1.2 c.c. Normal serum does not show this speed of toxification.

A mixture of distilled water and sensitized rat serum is rapidly toxified at 37 C., the speed increasing with the dilution. The dose containing 1 c.c. serum equivalent, incubated for 5 minutes, is fatally toxic (Table 137).

A mixture of horse serum and sensitized rat serum can be toxified at 37 C. in from 1 to 2 minutes, so that in dose of 2 to 3 c.c. of rat serum equivalent, it produces subacute death. The larger dose of 4 c.c. causes typical acute shock.

A mixture of distilled water and serum of rat sensitized to horse serum is likewise rapidly toxified at 37 C.; it may even be fatal in dose of 0.25 c.c. serum equivalent (Table 144).

Mixtures of diverse sera with their corresponding antisera are likewise promptly toxified at 37 C.

The normal rat serum when heated at 55 for 5 minutes, or at 50 for 17 minutes, becomes inactivated and no longer will produce poison when mixed with distilled water; it may yield some on treatment with agar.

The matrix which yields the poison is therefore readily destroyed by exposure to 50 C., or higher.

When distilled water is allowed to act on normal rat serum, the maximal speed of toxification appears to occur at about 45 C.; in 7 minutes it is possible to toxify such serum so that it is acutely fatal in 1 c.c. dose (Table 141).

The speed of poison-production in normal serum or in sensitized serum is the same as that which has been demonstrated for agar, trypanosomes, peptone, etc.

The in-vivo production of anaphylatoxin in specific shock, and its production, in vitro, in mixtures of sensitized sera and antigen or distilled water, is therefore an accomplished fact. The results contraindicate the theories of adsorption and of proteolysis.

The specific anaphylactic shock is the result of anaphylatoxin production, in corpore, consequent upon the inducing action of a body which is formed by the union, or otherwise, of antigen and its specific antibody. It is not necessary for this inducing body to exist as a visible precipitate, since substances in solution are capable of giving rise to anaphylatoxin. The specificity of the reaction concerns the production of this inducing substance, and not that of the poison; and further, the antigen is in nowise the source of the anaphylatoxin which is brought into being in shock.

X. ANAPHYLATOXIN AND AMINO-NITROGEN

P. H. DEKRUIF AND W. M. GERMAN

SYNOPSIS

INTRODUCTION

TECHNIC

AMINO-NITROGEN CONTENT OF NORMAL SERUM

ANAPHYLATOXIN AND AMINO-NITROGEN

AGAR ANAPHYLATOXIN

DISTILLED WATER-RAT SERUM ANAPHYLATOXIN

INULIN ANAPHYLATOXIN

SENSITIZED SERUM + ANTIGEN

SUMMARY

The majority of workers who have concerned themselves with the study of anaphylatoxin believe that proteolysis is in some way responsible for the formation of this poison. Friedberger and his pupils insist that the matrix of the toxin lies in the antigen used. It is their notion that the complement of normal guinea-pig serum plays the part of a proteoclastic enzyme, digesting the proteins of the antigen with the resulting formation of toxic albumoses.

Nathan¹ demonstrated that normal guinea-pig serum could be toxified with starch, while Bordet produced typical anaphylatoxin by mixing fresh serum with 0.5% agar-hydrogel. Unlike the bacteria which Friedberger used as antigen, these substances contain minimal quantities of albuminoid nitrogen. Friedberger,² however, stubbornly maintained this might still be the source of the poison. Friedberger's objection was finally set aside by Kopaczewski and Mutermilch,³ who produced anaphylatoxin by incubating guinea-pig serum with pectin, a nitrogen-free colloid. Bordet and Zunz⁴ further clinched the matter by obtaining a like result with pararabine.

The view now arose that anaphylatoxin resulted from serum autolysis, and that the bacteria and foreign colloids introduced served merely to uncover a latent proteoclastic enzyme, which then proceeded to attack the proteins of the serum itself. This theory has been enunciated most clearly by Jobling and Petersen,⁵ and by Bronfenbrenner.⁶

¹ Ztschr. f. Immunitätsf., 1913, 18, p. 636.

² Ibid., 18, p. 323.

³ Ibid., 1914, 22, p. 539.

⁴ Ibid., 23, p. 42.

⁵ Jour. Exper. Med., 1914, 19, p. 480.

⁶ Ibid., 1915, 21, p. 480.

The former authors extracted normal guinea-pig serum over a period of from 12 hours to several days with chloroform or ether. They found that serum so treated became toxic in minute doses for homologous animals. It was their idea that these solvents removed lipoidal antiferments, with the result that a protease was set free. Their results are to be questioned seriously, since N. R. Smith⁷ has shown that the method they employed does not free the serum entirely from the chloroform introduced, and that the residual amounts of chloroform are responsible for the toxic effects observed.

Bronfenbrenner incubated pregnant guinea-pig serum with boiled placenta and obtained a potent anaphylatoxin after 16 hours digestion. He tested the serum at the same time for dialyzable split products by the ninhydrin and biuret reactions. It appeared to him that the toxicity of the serum coincided with the appearance of these dialyzable bodies. Bronfenbrenner obtained first evidences of toxicity in 8 hours and found that maximal effect was to be observed in 16 hours. If this time were necessary for anaphylatoxin-production, it might be supposed that a ferment was concerned in the reaction. Such, however, is not the case. The production of anaphylatoxin may occur with extreme rapidity, and it is just this fact that makes it difficult to look upon this reaction as one of proteolysis.

Bordet and Zunz⁸ recognized the value of the Van Slyke nitrous acid method in the detection of proteolysis and applied it to the study of agar and pararabine anaphylatoxins. These investigators incubated guinea-pig serum for from 2 to 3 hours with these substances and observed some increase in the aliphatic amino-nitrogen. This increase was very small in the case of pararabine. In one instance, where the toxicity was very marked, an increase of only 0.9 mg. per 100 c.c. was observed. Bordet and Zunz show proper caution in the interpretation of their results. They conclude that the proteolysis may as well be a concomitant as a causative phenomenon.

The method of Van Slyke⁹ furnishes by far the most delicate and accurate instrument for determining protein cleavage, and we have utilized it exclusively in the experiments presented below. While it is plausible that there might be proteolysis in serum incubated over a period of 16 hours, it might be doubted that such a change could be

⁷ Jour. Lab. and Clin. Med., 1916, 1, p. 584.

⁸ Ztschr. f. Immunitätsf., 1914, 23, p. 49.

⁹ Jour. Biol. Chem., 1912, 12, p. 275; 1913, 16, p. 121; 1915, 23, p. 407.

detected in a few minutes. It has been our purpose to produce anaphylatoxin at a maximal speed, and to study any changes in quantity of serum amino-nitrogen that might accompany such production.

TECHNIC

The micro-apparatus of Van Slyke was used in all the determinations made. The serum-proteins were removed by precipitation with 10 volumes of 95% alcohol. To insure complete precipitation, this mixture was allowed to stand from 12 to 24 hours at the temperature of the room. The precipitate was then filtered off by suction and subsequently washed with a little alcohol. The washings were joined to the first filtrate. If the filtrate was not clear, as sometimes happened in the precipitation of dilute sera, gravity filtration was substituted for that by suction. The alcohol of the filtrate was then distilled off under reduced pressure at a boiling point of 38 C. and the water remaining was evaporated to dryness in a current of warm air, care being taken to avoid complete desiccation. The residue was taken up in 2 c.c. of distilled water and the determination made at once. Correction was made for nitrogen of reagents, for barometric pressure and for temperature. The reading on the gas-buret was then calculated to mg. of nitrogen per 100 c.c. of serum.

AMINO-NITROGEN CONTENT OF NORMAL SERUM

A series of preliminary determinations were made with normal rat serum, obtained by bleeding under ether with heart pipettes, immediate defibrination with a glass rod, and centrifugation of the clot-free blood at 8000 r.p.m. The same method was employed with all the material used in this work. The results of the series of preliminary tests with 7 different pools of serum are given in Table 147.

TABLE 147
AMINO-NITROGEN CONTENT OF NORMAL RAT SERUM

No.	Source	Amount of Serum Used (c.c.)	Amino-Nitrogen per 100 c.c. (mg.)
1	Rat	10	4.304
2	Rat	5	4.411
3	Rat	10	4.508
4	Rat	10	4.000
5	Rat	10	3.541
6	Rat	10	4.304
7	Rat	5	3.328

In the case of normal guinea-pig serum, we have found the variation to be slightly greater, that is, from 2 to 5 mg. per 100 c.c. The variation has no significance in subsequent experiments, since determinations on anaphylatoxic serum were invariably controlled by normal serum from the same pool.

ANAPHYLATOXIN AND AMINO-NITROGEN (AGAR ANAPHYLATOXIN)

Eight rats were bled in rapid succession, the blood defibrinated, pooled and centrifugated at 8000 r. p. m. It was iced for 1 hour before the experiment.

Five-tenths gm. of agar was dissolved in 100 c.c. of distilled water and autoclaved at 120 C. for 5 minutes, then gelled in cracked ice. The serum was then divided into 2 parts and treated as follows:

I. 14 c.c. serum, 38 C. + 3.5 c.c. agar-gel, 38 C., mixed for 1 min., incubated at 38 C. for 5 min., centrifuged at 3000 r. p. m. for 10 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

II. 12 c.c. serum, 38 C. + 3 c.c. 0.85% NaCl, incubated at 38 C. for 5 min.

Mixture divided..... { (a) 12.5 c.c. + 10 vol. alc., for Van Slyke.
(b) Balance tested for toxicity

The result is presented in Table 148.

TABLE 148
AMINO-NITROGEN CONTENT OF AGAR-RAT SERUM: 5 MIN. INCUBATION

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino- Nitrogen per 100 c.c. (mg.)
I. Serum + agar.....	5	180	1.0	3'20"	1.688
II. Serum control.....	5	192	1.0	Nil	1.07

It will be seen that while the portion treated with agar became toxic after 5 minutes' incubation, it showed actually less amino-nitrogen than the control. Duplicates of this test invariably gave the same general result, that is, a lowering of the amino-nitrogen content of the anaphylatoxin as compared with the control.

An attempt was now made to duplicate this result with guinea-pig serum. In this case, the incubation with agar was prolonged to 15 minutes, since guinea-pig serum has not the lability of that of the rat.

Three guinea-pigs were bled in the usual way, the blood pooled and centrifugated. It was used at once after centrifugation. Thirty c.c. of the pooled serum were divided into 2 portions, as follows:

I. 20 c.c. guinea-pig serum + 4 c.c. agar-gel were mixed 1 min. at room temperature, placed at 38 C. for 15 min., transferred at once to centrifuge and swung for 10 min. at 3000 r. p. m.

Supernatant divided... { (a) 10 c.c. precipitated for Van Slyke.
(b) Balance tested for toxicity

II. 10 c.c. guinea-pig serum + 2 c.c. 0.85 % NaCl incubated at 38 C. for 15 min., mixture precipitated at once for Van Slyke.

TABLE 149
AMINO-NITROGEN OF AGAR-GUINEA-PIG SERUM; FIFTEEN MINUTE CONTACT

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum + agar.....	15	190	3.0	3/5" Typical	1.925
II. Serum control.....	15	—	—	—	2.975

The result in the case of the guinea-pig serum will be seen to be analogous to that of rat serum, although the drop in the amino-acid of the anaphylatoxin is not as great as in the latter. In another experiment of a similar nature, the toxic serum gave a value of 2.663 as against 4.26 mg. per 100 c.c. for the control.

The drop in the amino-acid content of the anaphylatoxin as compared with the normal serum control occurs within 5 minutes after the time the agar is mixed with the serum. It would be interesting to note whether the same result would follow mixture and immediate centrifugation. The following experiment shows that this is the case. It indicates that mere mixing of the guinea-pig serum with agar-gel, without incubation, gives rise to a noticeable amount of anaphylatoxin.

I. Two preliminary control injections of normal serum.

II. 20 c.c. guinea-pig serum, room temperature + 4 c.c. agar-gel, room temperature, mixed 1 min., centrifugated 5 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

III. 10 c.c. guinea-pig serum + 2 c.c. 0.85 % NaCl stood room temperature 5 min., + 10 vol. alc. for Van Slyke

TABLE 150
AMINO-NITROGEN CONTENT OF AGAR GUINEA-PIG SERUM; NO INCUBATION

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum control.....	0	193	4.0	Nil	
	0	192	4.0	Nil	
II. Serum + agar.....	0	200	4.0	3/5" Typical	2.39
	0	195	4.0	Severe shock	
III. Normal serum + NaCl.....	0	—	—	—	3.82

Neither incubation nor any appreciable length of time is necessary to produce the amino-acid decrease in agar-treated serum. Again, the reaction of toxin-production is almost instantaneous, since the serum-agar mixture began to centrifuge within 1 minute after the 1-minute agitation in the flask. Kopaczewski and Mutermilch¹⁰ obtained a similar result. They, too, used guinea-pig serum with agar as the toxin inducer. The dose used in their experiments was 4.5 c.c. These authors observed severe shocks with such doses, even when the serum and agar were separately iced before mixture.

The results given were duplicated several times. When the agar and serum were separately incubated at 38 C. before mixture, the drop in amino-nitrogen was just as marked. It is worthy of note that the serum in this case was no more toxic, indeed even less so, than in the experiment tabulated above. If an enzyme were concerned in the toxin-production, one would expect the reverse to be true.

It was thought desirable to extend the preceding experiment with the addition of a sample incubated for 5 minutes at 38 C. The toxicity of such a mixture might well be greater than that of one not incubated. On the other hand, the foregoing tests would make it seem doubtful that there would be any parallel rise in amino-nitrogen. These expectations are shown to be justified by the following experiment.

A fresh pool of 55 c.c. of normal guinea-pig serum was used, together with the usual 0.5% agar-hydrogel.

I. 10 c.c. guinea-pig serum + 2 c.c. 0.85 % NaCl, (both room temperature), stood at room temperature 6 min., + 10 vol. alc. for Van Slyke

II. 20 c.c. guinea-pig serum + 4 c.c. agar-gel, (room temperature) mixed 1 min., centrifugated at 3000 r. p. m. for 6 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

III. 25 c.c. guinea-pig serum + 5 c.c. agar-gel, (room temperature) mixed 1 min., 38 C. 5 min., centrifugated for 6 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) Balance tested for toxicity

The result of this experiment is given in Table 151.

This experiment demonstrates that a marked rise in toxicity may occur with no corresponding rise in amino-nitrogen. On the contrary, the amino-nitrogen of the sample incubated for 5 minutes at 38 C. is slightly lower than in the case of the nonincubated agar-serum mixture.

We do not know the cause of this decrease in amino-nitrogen in the agar treated serum, although it seems most probable that some of the

¹⁰ Compt. rend. Soc. de biol., 1914, 77, p. 392.

TABLE 151
AMINO-NITROGEN CONTENT OF AGAR GUINEA-PIG SERUM; 5 MINUTES' INCUBATION

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Normal serum + NaCl.....	—	—	—	—	4.15
II. Serum + agar.....	0	200	4.0	Slight	3.66
	0	204	4.0	Slight	
III. Serum + agar.....	5	198	4.0	4' Typical	3.27
	5	200	4.0	Severe shock	

amino-acid may be adsorbed by the agar-gel. It has been shown by Hamburger¹¹ that agar can adsorb enzymes, and we have good reason to believe that serum proteins may also be in part taken up by this substance.

We have attempted to recover amino-acids from the precipitate of agar thrown down by centrifugation of the agar-serum mixtures. Our procedure was to take up the agar sediment in distilled water by means of a bulb pipette, shake the mixture vigorously for 10 minutes, and digest it at 38 C. for one-half hour. The mixture was then re-centrifuged at 8000 r.p.m. to remove all traces of agar, and the supernatant was concentrated in a current of warm air. Ten volumes of 95% alcohol were added to the concentrate. The fairly heavy flocculent precipitate which resulted showed the presence in the fluid of a considerable amount of serum protein. The usual technic of filtration and concentration was then carried out, followed by the Van Slyke determination. No amino-nitrogen was evolved.

It is possible that if there is an adsorption of amino-acid by the agar, the combination is so firmly fixed that distilled water will not break it.

Since Bordet and Zunz⁸ observed a distinct increase in amino-nitrogen, after incubating guinea-pig serum with agar for 3 hours at 38 C., we considered it likely that the preliminary drop might be followed by a rise if the incubation were continued. Experiments were made to detect such an increase.

¹¹ Arch. néer. d. sc. exact. et nat., 1908, 13, p. 428.

A pool of 40 c.c. of guinea-pig serum was divided into 2 portions and tested as follows:

- I. 20 c.c. guinea-pig serum, 38 C., + 4 c.c. agar-gel, 38 C., incubated for 2 hr., 30 min., at 38 C., then centrifugated at 3000 r. p. m. for 10 min.
 Supernatant divided... $\left\{ \begin{array}{l} \text{(a) 10 c.c. + 10 vol. alc., for Van Slyke} \\ \text{(b) Balance tested for toxicity} \end{array} \right.$
- II. 15 c.c. guinea-pig serum, 38 C., + 3 c.c. 0.85% NaCl, 38 C., 2 hr., 30 min.
 12 c.c. of mixture
 + 10 vol. alc., for Van Slyke
 10 c.c. actual
- The result of the experiment is given in Table 152.

TABLE 152
 AMINO-NITROGEN CONTENT OF GUINEA-PIG SERUM; TWO HOURS, THIRTY MINUTES' INCUBATION

Treatment of Serum	Time at 38 C.	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum + agar.....	2 hr. 30'	195	2.0	Near-kill	3.497
II. Serum + NaCl.....	2 hr. 30'	—	—	—	3.579

In this case the amount of amino-nitrogen is distinctly greater, as compared with the control, than in the experiments where short agar contact was employed. It would be too much to conclude that a distinct rise in amino-nitrogen had occurred, since no brief contact sample was available in this experiment. An experiment which should include the latter test was therefore made.

A pool of fresh guinea-pig serum was made and divided into 4 parts as follows:

- I.—1. 20 c.c. guinea-pig serum, 38 C., + 4 c.c. agar-gel, 38 C., incubated at 38 C., 15 min., then centrifugated at 3000 r. p. m. for 10 min.
 Supernatant divided... $\left\{ \begin{array}{l} \text{(a) 10 c.c. + 10 vol. alc., for Van Slyke} \\ \text{(b) Balance tested for toxicity} \end{array} \right.$
2. 15 c.c. guinea-pig serum, 38 C., + 3 c.c. 0.85% NaCl, 38 C., mixture 38 C., 15 min.
 12 c.c. of mixture
 + 10 vol. alc., for Van Slyke
 10 c.c. serum
- II.—1. 20 c.c. guinea-pig serum, 38 C., + 4 c.c. agar-gel, 38 C., incubated at 38 C., 3 hr., 30 min., centrifuged at 3000 r. p. m., 10 min.
 10 c.c. of supernatant + 10 vol. alc., for Van Slyke
2. 15 c.c. guinea-pig serum, 38 C., + 3 c.c. 0.85 % NaCl, 38 C., incubated at 38 C., 3 hr., 30 min.
 12 c.c. of mixture
 + 10 vol. alc., for Van Slyke
 10 c.c. serum

The result of this experiment is given in Table 153.

TABLE 153
AMINO-NITROGEN CONTENT OF AGAR GUINEA-PIG SERUM; LONG AND SHORT INCUBATION

Treatment of Serum	Time at 38 C.	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. (a) Serum + agar	15'	202	3.0	3'45"	3.715
	15'	210	2.0	4'20"	
(b) Serum + NaCl	15'				3.23
II. (a) Serum + agar	3 hr. 30'				4.055
(b) Serum + NaCl	3 hr. 30'				4.013

This experiment shows an increase of 0.3 mg. per 100 c.c. in the agar-treated serum, while the control shows an increase of 0.8 mg. over the sample incubated for 15 minutes. It would appear, then, that increase in amino-nitrogen was hardly appreciable, even when the incubation was carried on for from 2 to 3 hours.

A more complete experiment of this kind was now performed, in which incubations were made over a period of 24 hours. The entire experiment was carried out with rigid aseptic precautions. A single pool of 160 c.c. of serum was used. The successive samples of the agar-treated serum and of the controls were removed, each from its flask, at the intervals indicated in outline of experiment.

I. 10 c.c. guinea-pig serum + 10 vol. 95 % alc., for Van Slyke

II. 50 c.c. guinea-pig serum + 10 c.c. 0.85 % NaCl, 38 C., incubation in sterile flask

12 c.c. portions

removed at 15 min., 4 hr., 8 hr., 12 hr., 24 hr. each + 10 c.c. serum 10 vol. 95 % alc., for Van Slyke

III. 100 c.c. guinea-pig serum + 20 c.c. agar-gel, 38 C., incubation in sterile flask

24 c.c. portions

removed at 15 min., 4 hr., 8 hr., 12 hr., 24 hr., each 20 c.c. serum centrifuged 3000 r. p. m., 6 min.

Supernatant divided... { (a) 10 c.c. + 10 vol. alc., for Van Slyke
(b) 10 c.c. for m. l. d. tests

The only inconsistency in this series of determinations is to be found in the 12 hour test of II. Here we found an unaccountable drop of 2 mg. per 100 c.c. This is probably due to some unobserved technical error. The conclusion to be drawn from this series of tests reveals the usual early slight decrease of the amino-nitrogen of the

TABLE 154
AMINO-NITROGEN CONTENT AND TOXICITY; AGAR GUINEA-PIG SERUM; TWENTY-FOUR-HOUR
INCUBATION

Treatment of Serum	No.	Time at 38 C.	Weight	Intravenous Injection (c.c.)	Result	Amino- Nitrogen per 100 c.c. (mg.)
I. Serum control		0	—	—	—	4.28
II. Serum + NaCl	1	15'				3.90
	2	4 hr.				4.00
	3	8 hr.	—	—	—	4.55
	4	12 hr.				2.42*
	5	24 hr.				4.87
III. Serum + agar	1	15'	205	2.0	3'	
	2	15'	210	1.0	Slight	3.47
	3	15'	205	1.5	Severe	
	4	4 hr.	172	1.5	Slight	
	5	4 hr.	200	2.0	Fair	3.44
	6	4 hr.	202	2.5	Fair	
	7	4 hr.	195	3.0	3'40"	
	8	8 hr.	210	2.5	3'	
	9	8 hr.	212	2.0	4'40"	3.25
	10	8 hr.	202	1.5	Near-kill	
	11	8 hr.	180	1.5	Very severe	
	12	12 hr.	190	1.5	Fair	
	13	12 hr.	195	2.0	3'30"	3.58
	14	12 hr.	190	2.0	Severe	
	15	24 hr.	200	2.0	Light	3.85
	16	24 hr.	175	3.0	3'	

* Drop due to technical error.

anaphylatoxin as compared with the control. There is a slight rise in the values of both control and toxin in 24 hours, but the amino-nitrogen of the anaphylatoxin never attains the amount of that of the control. On the other hand, the rise in toxicity is very sudden, practically attaining its maximum in 15 minutes. It is possible that there is a slight increase in the 8-hour test (III, 10), but this apparent increase in lethal power may as well be due to the use of a hypersusceptible guinea-pig. Chart 17 indicates the relationship between toxicity and amino-acid content of anaphylatoxin.

ANAPHYLATOXIN AND AMINO-NITROGEN (DISTILLED WATER-RAT SERUM ANAPHYLATOXIN)

Agar-hydrogel was used as the inducing agent in all the preceding experiments. In Part IX, it has been shown that rat serum can be made anaphylatoxic by simple dilution with distilled water, accom-

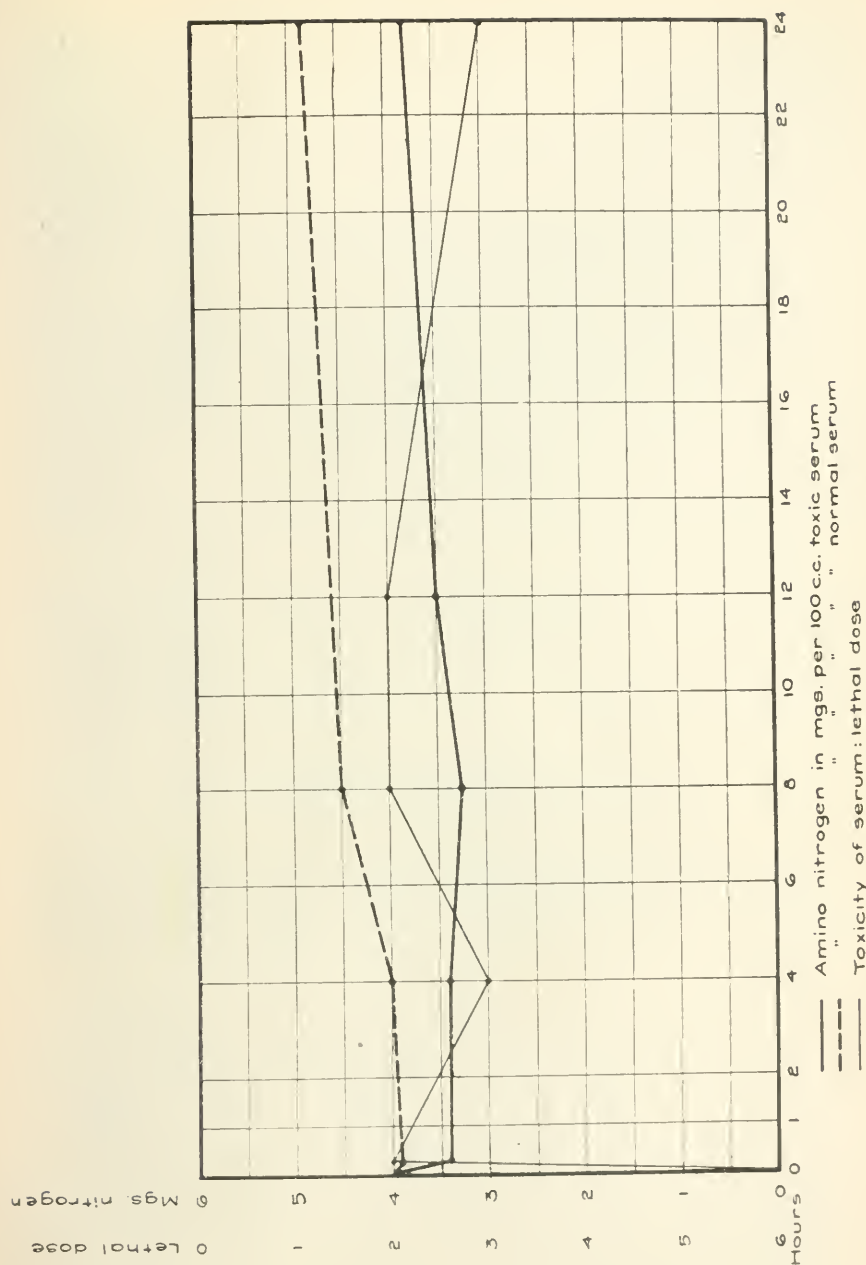


Chart 17.—Relation of Amino-nitrogen content to the toxicity of the Agar Guinea-pig serum (Table 154).

panied by incubation at 38 C. Sensitized rat serum is especially easy to toxify by this method, although similar results can be obtained by the use of normal serum. The only difference between the two lies in the fact that the former toxifies with greater rapidity. For example, if the serum of an egg-white sensitive rat is diluted in a proportion of 1:6 with distilled water, it becomes fatally toxic after 5 minutes at 38 C. Normal serum diluted in a similar manner requires 30 minutes of incubation to attain this toxicity.

Parallel determinations of toxicity and amino-nitrogen would be especially interesting in this type of anaphylatoxin-production, since no foreign element is introduced, the dilution being the only change. The injections of the diluted sera were made rather slowly (from 30 to 60 seconds), since too rapid injections of fluids of a low electrolyte content have a disturbing influence. Very severe effects might follow the sudden introduction of 6 c.c. of distilled water into the circulation of a 200-gm. guinea pig.

In the protocols presented, sensitive rat serum will be spoken of as 'rat > e.w. serum.' All the sensitive serum used in these experiments was obtained by intravenous injection of rats with 1% solutions of Kahlbaum's egg-white. Sensitization of the rats was carried out 38 days before exsanguination. The sera were pooled before use.

- I. (a) 4 c.c. rat > e. w. serum + 24 c.c. distilled H₂O mixed 15 sec., + 10 vol. 95 % alc. for Van Slyke (control)
- (b) 1 c.c. rat > e. w. serum + 6 c.c. distilled H₂O mixed 15 sec., injected at once (control)
- (c) 1 c.c. rat > e. w. serum, 38 C., + 6 c.c. dist. H₂O, 38 C. mixed 15 sec., inc. 38 C., 5 min., injected at once
- (d) 4 c.c. rat > e. w. serum, 38 C., + 24 c.c. dist. H₂O, 38 C. mixed 15 sec., 38 C., 5 min. + 10 vol. alc., Van Slyke

TABLE 155
AMINO-NITROGEN CONTENT OF DILUTED SENSITIZED RAT SERUM

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. (a) Serum + H ₂ O	0	—	—	—	2.03
(b) Serum + H ₂ O	0	185	1.0	Nil	
(c) Serum + H ₂ O	5	187	1.0	3'10"	
(d) Serum + H ₂ O	5	—	—	—	3.45

The toxicity will be seen to have reached a 1 c.c. intensity after 5 minutes at 38 C. At the same time a rise of 1.4 mg. per 100 c.c. in amino-nitrogen took place. At first glance, one might infer that the toxicity was related to this rise. The following experiment shows that this is not true.

The conditions of the previous experiment were duplicated with the exception that pooled normal rat serum was substituted for the rat >e.-w. serum. Further, successive mixtures of the normal serum and distilled water were incubated over increasing lengths of time until the killing power of the mixtures attained a strength of 1 c.c. serum equivalent. When this incubation time had been ascertained, the Van Slyke mixture (C) was incubated for a corresponding length of time. Here, again, as in the preceding tests, the injections were made very slowly.

- I. (a) 4 c.c. normal rat serum + 24 c.c. distilled H₂O mixed 15 sec., + 10 vol. 95 % alc., Van Slyke .
 (b) 1 c.c. normal rat serum + 6 c.c. dist. H₂O mixed 15 sec., injected at once, control
 (b₁) 1 c.c. normal rat serum, 38 C. + 6 c.c. dist. H₂O, 38 C., mixed 15 sec., 38 C., 10 min., injected
 (b₂) like (b₁), 20 min. 38 C.
 (b₃) like (b₁), 30 min. 38 C.
 (c) 4 c.c. normal rat serum + 24 c.c. distilled H₂O, mixed 15 sec., 38 C., 30 min., + 10 vol. alc., Van Slyke

TABLE 156
AMINO-NITROGEN CONTENT OF DILUTED NORMAL RAT SERUM

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. (a) Serum + dist. H ₂ O	0	—	—	—	1.00
(b) Serum + dist. H ₂ O	0	200	1.0	Nil	
(b ₁) Serum + dist. H ₂ O	10	200	1.0	Slight	
(b ₂) Serum + dist. H ₂ O	20	185	1.0	Fair	
(b ₃) Serum + dist. H ₂ O	30	188	1.0	0.15	
(c) Serum + dist. H ₂ O	30	—	—	—	2.84

While an increase in amino-nitrogen occurred after incubation of the diluted rat > e.w. serum, a decrease will be observed to have taken place in the normal serum similarly diluted and incubated to a 1 c.c. killing power. These results are not fortuitous or due to technical variation. For in another series of tests, the amino-nitrogen of a rat > e.w. serum diluted 1:6 gave a figure of 5.51 before incubation, and 6.81 after 10 minutes at 38 C. A control of similarly diluted normal serum gave 6.09 before incubation, and 4.75 after 10 minutes at 38 C.

We think it desirable to extend these experiments and plan to do so as soon as a considerable supply of large rats becomes available. The present experiments are not extensive enough to warrant an explanation of this peculiar difference between normal and sensitive serum. They do show, however, that toxification does not depend on small increases or decreases in amino-nitrogen content.

ANAPHYLATOXIN AND AMINO-NITROGEN (INULIN ANAPHYLATOXIN)

Nathan¹² has demonstrated that the polysaccharid, inulin, produces anaphylatoxin in normal guinea-pig serum and that its ability to do so depends upon its physical state. An inulin-suspension produces the toxin readily, while a solution of the same substance fails to initiate such toxicity. The suspensions used in the following work were prepared by rubbing up, in the cold, 5 gm. of Kahlbaum's inulin with 100 c.c. 0.85% NaCl solution.

The experiments are, in general, of the same type as those made with agar-hydrogel. Toxicity tests of the serum were made parallel to the Van Slyke determinations. Before proceeding with the anaphylatoxin experiments, Van Slyke determinations were made of the inulin-suspension. This was found to contain an appreciable amount of amino-nitrogen. The procedure used in these determinations was identical with that in the serum work. A series of tests gave an average of from 3 to 4 mg. per 100 c.c. for 5% inulin-suspensions.

Although the amounts of inulin suspension used in anaphylatoxin-production were small, the amino-nitrogen content could not be neglected. Consequently, the values are expressed in terms of total nitrogen per 100 c.c. of mixture, rather than per 100 c.c. of serum.

¹² Ztschr. f. Immunitätsf., 1914, 23, p. 204.

I. 40 c.c. guinea-pig serum + 4.0 c.c. 5 % inulin, mixed 1 min.
5.5 mixture

(a) ————— removed, centrifuged 3000 r. p. m., supernatant + 10 vol.
5 c.c. serum alc., Van Slyke, balance at 38 C.

(b) After 30 min. at 38 C., 15 c.c. removed, centrifuged 3000 r. p. m.

Supernatant..... { 1. 5.4* + 10 vol. alc., Van Slyke
 { 2. Balance tested for toxicity

(c) After 90 min. at 38 C., remainder centrifuged 3000 r. p. m.

Supernatant..... { 1. 5.4* + 10 vol. alc., Van Slyke
 { 2. Balance tested for toxicity

* 5.4 c.c. is volume of supernatant when 5.5 c.c. are centrifuged, and is equivalent to 5.0 c.c. of serum.

The result of this experiment is given in Table 157.

TABLE 157
AMINO-NITROGEN CONTENT OF INULIN GUINEA-PIG SERUM

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. (a) Serum + inulin	0	—	—	—	4.59
(b) Serum + inulin	30	180	2.7	Nil	3'40"
	30	195	4.5		
(b ₁) Serum + inulin	30	—	—	—	6.49
(c) Serum + inulin	90	180	2.7	4'	3'40"
	90	175	1.8		
	90	185	1.3	Nil	
(c ₁) Serum + inulin	90	—	—	—	5.95

After 30 minutes at 38 C., it will be noted that the toxicity is marked, but not at a maximum. Coincident with the toxin increase, the amino-nitrogen has risen 1.9 mg. per 100 c.c. of mixture. At the end of 90 minutes, at 38 C., the toxicity has increased to a great extent, 1.8 c.c. producing a fatal shock. The amino-nitrogen has at the same time dropped 0.5 mg. per 100 c.c. While there is a slight rise in amino-nitrogen, together with some toxicity, after an incubation of 30 minutes, the nitrogen increase fails entirely to keep pace with the intensification of the toxic state.

The rise noted in the case of the guinea-pig anaphylatoxin might lead one to conclude that at least the initiation of toxicity is accompanied by proteolysis. The following experiment with rat serum shows this to be untrue.

A pool of fresh rat serum was used in this experiment and a new suspension of inulin (5%) was made.

- I. (a) 1 c.c. rat serum + 0.1 c.c. 5 % inulin mixed 30 sec., centrifugated at once, 3000 r. p. m., tested for toxicity
- (b) 4 c.c. rat serum + 0.4 c.c. 5 % inulin mixed 30 sec., centrifugated at once, 3000 r. p. m., supernatant + 10 vol. 95 % alc., for Van Slyke
- II. (a) 2 c.c. rat serum, 38 C., + 0.2 c.c. inulin mixed 30 sec., placed at 38 C., 2 min., 30 sec., centrifugated 3000 r. p. m., 7 min., supernatant tested for toxicity
- (b) 4 c.c. rat serum, 38 C., + 0.4 c.c. inulin mixed 30 sec., placed at 38 C., 2 min., 30 sec., centrifugated 3000 r. p. m., 7 min., supernatant + 10 vol. 95 % alc., for Van Slyke
- III. (a) 2 c.c. rat serum, 38 C., + 0.2 c.c. inulin mixed 30 sec., placed at 38 C., 7 min., centrifugated 3000 r. p. m., 7 min., supernatant tested for toxicity
- (b) 4 c.c. rat serum, 38 C. + 0.4 c.c. inulin mixed 30 sec., placed at 38 C., 7 min., centrifugated 3000 r. p. m., 7 min., supernatant + 10 vol. 95 % alc., for Van Slyke

The entire experiment covered less than an hour of time, so that any variation in amino-nitrogen, on account of time in I (b), II (b), or III (b), would be improbable.

The results are given in Table 158 and Chart 18.

TABLE 158
AMINO-NITROGEN CONTENT OF INULIN RAT SERUM

Treatment of Serum	No.	Time at 38 C.	Weight	Intravenous Injection (c.c.)	Result	Amino-Nitrogen per 100 c.c. (mg.)
I. Serum + inulin						
(a)	1	0	205	1.0	Nil	
(b)		0	—	—	—	3.02
II. Serum + inulin						
(a)	2	2'30"	205	1.0	4'	
(b)	3	2'30"	190	0.5	Near-kill	
		2'30"	—	—	—	2.67
III. Serum + inulin						
(a)	4	7'	206	0.5	3'25"	
	5	7'	185	0.25	3'30"	
(b)	6	7'	193	0.20	Slight	
		7'	—	—	—	2.73

It is necessary to place the mixture at 38 C. for but 2½ minutes to obtain a killing power of between 0.5 and 1.0 c.c. Despite this, the amino-nitrogen has fallen off distinctly. Furthermore, in the brief incubation time of 7 minutes, the serum has reached an extreme

toxicity, yet the amino-nitrogen, III (b), is hardly larger in amount than that in II (b), and is certainly less than that in the control, I (b). See Chart 18.

To sum up, both guinea-pig and rat serum can be made anaphylatoxic by incubation with inulin suspension. Guinea-pig serum shows a slight early rise in amino-nitrogen, along with a moderate early toxicity. Subsequent intensification is not accompanied by a corresponding increase of amino-acid. The behavior of rat serum is totally different, and presents still stronger evidence of a dissociation of the phenomena of toxification and proteolysis. For while a maximum toxicity appears, the amino-nitrogen becomes less, as in the case of agar.

ANAPHYLATOXIN AND AMINO-NITROGEN (SENSITIZED
SERUM + ANTIGEN)

Sensitive guinea-pig or rat serum can be made anaphylatoxic by incubation with the antigen used in sensitizing. This is also true of rabbit serum. We have produced anaphylatoxin in this manner with guinea-pig > egg-white and rabbit > egg-white sera and have made parallel tests of the amino-nitrogen. If a specific proteoclastic ferment were formed in response to the injection of a foreign protein, and if the toxicity subsequent to the bringing together of the serum of the injected animal, and the specific antigen were due to proteolysis of the latter, a large increase in amino-nitrogen should accompany the toxicity. The following experiments show that this is not the case. In fact, there is no evidence whatever of proteolysis, either of the serum or the antigen. At the same time, the serum in question becomes toxic to marked degree.

The rabbit used as source of the sensitive serum was made hyper-sensitive by 6 heavy intravenous injections of 4% egg-white, the dose-ranging from 2 to 8 c.c. Kahlbaum's egg-white was used as an antigen in this experiment (1% in 0.85% NaCl solution).

- I. Test of toxicity of rabbit > egg-white serum
- II. (a) 2 c.c. rabbit > e.-w. serum + 0.2 egg-white, 38 C., 5 min.
 (b) 2 c.c. rabbit > e.-w. serum + 0.2 c.c. egg-white, 38 C., 10 min.
 (c) 2 c.c. rabbit > e.-w. serum + 0.2 c.c. egg-white, 38 C., 15 min.
 (d) 2 c.c. rabbit > e.-w. serum + 0.2 c.c. egg-white, 38 C., 20 min.
 (d₁) 2 c.c. rabbit > e.-w. serum + 0.2 c.c. egg-white, 38 C., 26 min.
- III. (a) 4 c.c. rabbit > e.-w. serum + 0.4 c.c. egg-white, mixed 30 sec., mixture + 10 vol. 95 % alc., for Van Slyke
 (b) Like (a), but incubated 38 C., 26 min. after mixture, then + 10 vol. alc.
 Injection dose, 2.2 c.c. of mixture, equivalent to 2 c.c. of serum.

TABLE 159
 AMINO-NITROGEN CONTENT OF RABBIT > EGG-WHITE SERUM

Treatment of Serum	Time at 38 C. (min.)	Weight	Intravenous Injection (c.c.)	Result	Amino- Nitrogen per 100 c.c. (mg.)
I. Rabbit > e.-w. serum (control of toxicity)	0	205	1.0	Nil	
	0	190	2.0	Slight	
	0	206	2.5	Slight	
	0	206	3.0	Moderate	
II. Rabbit > e.-w. serum + egg-white	(a) 5	202	2.0	Nil	
	(b) 10	190	2.0	Fair	
	(c) 15	195	2.0	Fair	
	(d) 26	195	2.0	S ³⁰	
	(d ₁) 26	196	2.0	Near-kill	
III. Rabbit > e.w. serum + egg-white	(a) 0	—	—	—	4.18
	(b) 26	—	—	—	2.003

The injections of sensitive serum not mixed with egg-white (I) serve as controls for the injections II (a), (b), (c), (d), and (d₁). The serum used, as would be expected from the many injections of egg-white made, had a very powerful precipitating action. For example, mixture II (a), after 5 minutes incubation, showed a heavy precipitate. It is possible that, here, as in the case of the agar-anaphylatoxin, the decrease in amino-nitrogen after incubation may be due to adsorption phenomena. The Van Slyke determinations, III (a) and (b) show clearly the absence of proteolysis in this case. At the same time, the toxicity of the serum egg-white mixtures attained a marked intensity after 26 minutes at 38 C. It may be remarked here that rabbit serum anaphylatoxin rarely reaches a higher concentration in regard to toxicity.

Similar experiments made with guinea-pig > egg-white serum yielded a like result. In this case, however, the guinea-pigs received but 1 injection of 1% egg-white. It required 60 minutes at 38 C. to toxify a mixture of 4 c.c. serum plus 1 c.c. of egg-white. A similar mixture, injected at once, without incubation, produced no effect. Its amino-nitrogen value was 1.35 mg. per 100 c.c. of mixture. Successive tests of 4 plus 1 mixtures were made after incubation at 5, 15, 30, and 60 minutes, respectively. These mixtures produced progressively heavier shocks, the last one (60 minutes) killing the recipient in 4 minutes and 10 seconds. The amino-nitrogen value of a 4 plus 1 mixture, incubated at 38 C. for 60 minutes was 1.25 per 100 c.c.

To sum up, a decrease rather than an increase in amino-nitrogen is

noticed when sensitive sera are incubated with their specific antigens. It should be borne in mind that none of these mixtures are incubated over long periods. They were kept at 38 C. only long enough to acquire marked toxicity, since it was our object only to find whether such toxification would be accompanied by proteolysis.

Careful search has failed to disclose any evidence that would connect digestion of serum proteins with the formation of anaphylatoxin. In 2 or 3 instances, slight increases in amino-nitrogen have been detected at times when a serum was also anaphylatoxic. But in each of these cases, the use of another inducing agent with the same serum has given rise to toxicity without increase in amino-nitrogen (Tables 157 and 158). In cases where a moderate toxicity was accompanied by a slight amino-acid increase, intensification of that toxicity might occur with no further increase, indeed, at times, even a drop, in amino-nitrogen (Table 157).

Dale¹³ has pointed out, in his work on the anaphylactic reaction of plain muscle, that the time relation of the reaction places a serious obstacle in the way of the ferment conception of anaphylaxis. The reaction is as rapid as in the case of application of a direct stimulant. We believe the toxification of normal serum to be a reaction identical with that occurring in the body cells and fluids during anaphylactic shock, and in the reaction studied, the same explosive effect is observed. In this connection, we wish to present an illustration of the time relation in anaphylatoxin-production.

Table 160 indicates the rise in toxicity of rat serum incubated with 5% inulin-suspension in a proportion 10:1. The table is a composite of a series of experiments, all of which were made with the same pool of rat serum and the same suspension of inulin. The deaths were all acute.

TABLE 160
SPEED OF INCREASE IN TOXICITY OF INULIN RAT SERUM

No.	M.l.d. 200 gm. g.p.	Time at 38 C.
1	0.50	20-30"
2	0.25	7"
3	0.20	17"
4	0.15*	20"
5	0.20	60"
6	0.20	28"

* To our knowledge the most potent anaphylatoxin ever obtained.

¹³ Jour. Pharmacol. and Exper. Therap., 1913, 4, p. 16"

Chart 18 shows clearly the curve of toxicity. The degree of toxicity is plotted on the ordinates. The abscissae represent the time in minutes. The toxicity of the rat serum before mixture with the inulin was in all probability not less than 6 c.c. for a 200-gm. guinea-pig.

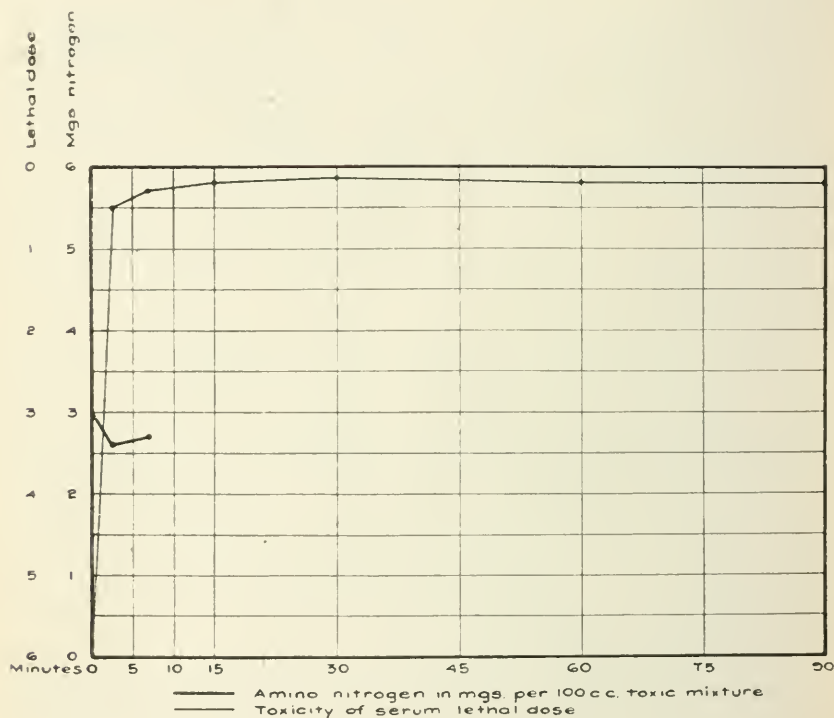


Chart 18.—Relation of Amino-nitrogen Content to the Toxicity of Inulin Rat Serum (Tables 158 and 160).

As Bayliss¹⁴ points out, the digestion of raw serum by trypsin is very slow. It is a matter of hours before the reaction begins to become noticeable. Those who assume that a trypsin-like ferment is operating here will find it difficult to reconcile this curve with that characteristic of tryptic action in raw serum.

Table 161 gives a summary of the experiments with anaphylatoxin. In the majority of these, agar was used as the inducing agent; in others, inulin, distilled water, or antigen were employed.

¹⁴ The Nature of Enzyme Action, 1914, p. 128.

TABLE 161
SUMMARY OF AMINO-NITROGEN VALUES IN NORMAL AND TOXIC SERA

No.	Source	Time at 35 C.	Lethal Dose per 200 gm. (c.c.)	Amino-Nitrogen per 100 c.c. (mg.)	
				Toxin	Control
1	Guinea-pig	0	4.0	2.39	3.82
2	Guinea-pig	0	—	3.66	4.16
3	Guinea-pig	1'	—	3.69	5.19
4	Guinea-pig	5'	—	3.27	4.16
5	Guinea-pig	15'	—	2.66	4.26
6	Guinea-pig	15'	3.0	1.92	2.47
7	Guinea-pig	15'	2.0	3.71	3.23
8	Guinea-pig	15'	2.0	3.47	3.90
9	Guinea-pig*	30'	4.5	6.49	4.5
10	Guinea-pig†	1 hr. 30'	1.8	5.95	4.5
11	Guinea-pig	2 hr. 30'	3.0	2.49	3.59
12	Guinea-pig	3 hr. 30'	3.0	4.08	4.01
13	Guinea-pig	4 hr.	3.0	3.44	4.09
14	Guinea-pig	8 hr.	2.0	3.25	4.55
15	Guinea-pig	12 hr.	2.0	3.52	2.42‡
16	Guinea-pig	24 hr.	3.0	3.85	4.87
17	Rat	2'30"	0.5	2.67	3.02
18	Rat	5'	1.0	1.68	4.67
19	Rat	5'	0.5	3.69	4.12
20	Rat†	7'	0.25	2.75	2.02
21	Rat‡	5'	1.0	3.45	2.0
22	Rat‡	10'	—	6.81	3.51
23	Rat‡	10'	—	4.75	6.09
24	Rat‡	30'	1.0	2.84	3.67
25	Rabbit§	26'	2.0	2.00	4.58
26	Guinea-pigs§	60'	4.0	1.25	1.65

* Inulin.

† Technical error.

‡ Distilled water.

§ Antigen.

SUMMARY

By the use of Van Slyke's method for the determination of aliphatic amino-nitrogen, we have attempted to discover a relationship between serum autolysis and anaphylatoxin-production. No relationship between these phenomena has been found.

Rat and guinea-pig sera, toxified rapidly by the addition of 5% agar-hydrogel, show a marked decrease in amino-nitrogen as compared with controls from the same pool of serum.

This decrease takes place at once upon addition of agar to serum. After this preliminary drop in amino-nitrogen, a very gradual rise to a value not exceeding that of the control may take place. Maximal toxicity appears before this increase begins to be noticeable.

The cause of this drop in amino-nitrogen in serum treated with agar is not known, but is probably to be referred to adsorption of amino-acid by the agar. Attempts to recover this supposedly adsorbed amino-acid have failed.

A similar drop in amino-nitrogen along with a marked increase in toxicity is to be observed in the case of rat serum incubated with inulin. Guinea-pig serum treated in a like manner shows a slight increase in amino-acids, which, however, does not keep pace with the increase in toxicity.

GENERAL INDEX

GENERAL INDEX

A

	PAGE
Agar anaphylatoxin: guinea-pig serum - - - - -	536
Agar anaphylatoxin: rat serum - - - - -	589
Agar anaphylatoxin: rabbit serum - - - - -	566
Agar, effect of intravenous injection of - - - - -	629
Allergic skin reaction in pneumonia - - - - -	344
Amino-nitrogen and anaphylatoxin - - - - -	833
Anaphylactic shock, specific - - - - -	776
Anaphylatoxin and amino-nitrogen - - - - -	833
Anaphylatoxin and anaphylaxis 499, 536, 566, 589, 629, 618, 657, 717, 776.	833
Anaphylatoxin, effect of multiple doses of - - - - -	618
Anaphylatoxin, peptone - - - - -	657
Anaphylatoxin, trypanosome - - - - -	499
Antibodies, nonspecific, passive transference of - - - - -	145
Antigen, preparation and properties of, for intracutaneous typhoidin reaction - - - - -	357

B

Bactericidal action of substances in vitro, methods of determination in relation to chemotherapy - - - - -	293
Bacillus anthracis-symptomatici and allied organisms with respect to gas production, comparative study of - - - - -	151
Bacterial infections, chemotherapy of - - - - -	293
Bacteriology of pemphigus neonatorum - - - - -	86
BAEHR, GEORGE, and PLOTZ, HARRY. Blood culture studies on typhus exanthematicus in Serbia, Bulgaria, and Russia - - - - -	201
BASINGER, H. RAYMOND. The alleged detoxicating power of the thyroid gland - - - - -	131
Blood culture studies on typhus exanthematicus - - - - -	201
BOYD, MARK F. A small contact epidemic of typhoid fever - - - - -	109
Bulgaria, blood culture studies on typhus exanthematicus in - - - - -	201

C

Carriers, diphtheria, virulence of bacilli from - - - - -	125
CARY, WILLIAM E. Virulence and toxin-formation in B. diphtheriae - - - - -	244
Chemotherapy of bacterial infections - - - - -	293
Chemotherapy of experimental trypanosomiasis - - - - -	10
CHRISTIANSEN, C. R. See MEYER.	
Cinchona derivatives, action on pneumococci in vitro - - - - -	272, 333
Cinchona derivatives, in experimental pneumococcus infection - - - - -	313
COHEN, MYER SOLIS-. Tuberculin hypersensitiveness as determined by intracutaneous tests of different degrees - - - - -	233

COHEN, S. SOLIS-. See KOLMER.

COHEN, S. SOLIS-, KOLMER, JOHN A., and HEIST, GEORGE D. Germicidal action and specific bacteriotropism of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchona derivatives on pneumococci in vitro. Studies in pneumonia, I - - - - - 272

COHEN, S. SOLIS-, KOLMER, JOHN A., and HEIST, GEORGE D. The protective and curative value of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchona derivatives in experimental pneumococcus infections. Studies in pneumonia, III - - - - - 313

D

Dale method, typhus antibodies demonstrable by - - - - - 99

DEKRUIF, P. H. See NOVY, F. G.

DEKRUIF, P. H. Anaphylatoxin and anaphylaxis. VIII. The primary toxicity of normal serum - - - - - 717

DEKRUIF, P. H., and GERMAN, W. M. Anaphylatoxin and anaphylaxis. X. Anaphylatoxin and amino-nitrogen - - - - - 833

DENZER, BERNARD S., and OLITSKY, PETER K. Studies on immunity in typhus exanthematicus with reference to the antibodies in man and guinea-pig demonstrable by the Dale method - - - - - 99

DENZER, BERNARD S. See OLITSKY.

Diarrhea, acute, epidemic of - - - - - 140

Diphtheria bacillus, virulence and toxin-formation in - - - - - 244

Diphtheria bacilli, virulence of, from patients and carriers - - - - - 125

Disinfection of swimming pools - - - - - 1

Dysentery, mild, epidemic of acute diarrhea approaching - - - - - 140

E

EBERSON, FREDERICK, and TEH, WU LIEN. Transmission of pneumonic and septicemic plague among marmots - - - - - 170

EBERSON, FREDERICK. Plague poisons and virulence - - - - - 180

Ethylhydrocuprein, action on pneumococci in vitro - - - - - 272, 333

Ethylhydrocuprein in experimental pneumococcus infection - - - - - 313

Etiology of acute poliomyelitis - - - - - 113

F

FALLS, FREDERICK HOWARD. The bacteriology of pemphigus neonatorum 86

G

Gas production, comparative study of anthracis-symptomatici and allied organism with respect to - - - - - 151

GERMAN, W. M. See DE KRUIF, P. H.

Gold chlorid, treatment of experimental tuberculosis combined with taurin 442

Guinea-pig serum, agar anaphylatoxin of - - - - - 536

Guinea-pigs, treatment of experimental tuberculosis in, with taurin, gold chlorid and sodium oleate - - - - - 442

H

PAGE

- HEIST, GEORGE D. See COHEN and KOLMER.
 Hemolysis, effect of chemical substances on - - - - - 185
 Hypersensitiveness, cutaneous, relation to immunity and infection - - 424
 Hypersensitiveness, tuberculin, determined by intracutaneous tests of different degrees - - - - - 233

I

- Infections, bacterial, chemotherapy of - - - - - 293
 Infection, relation of cutaneous hypersensitiveness to - - - - 424
 Immunity in typhus exanthematicus - - - - - 99
 Immunity, relation of cutaneous hypersensitiveness to - - - - 424

J

- JORDAN, EDWIN O. The differentiation of the paratyphoid-enteritidis group, I - - - - - 457

K

- KELSO, R. F. See SADLER.
 KOLMER, JOHN A., COHEN, S., SOLIS-, and HEIST, GEORGE D. Various methods of determining the bactericidal action of substances in vitro and their relation to the chemotherapy of bacterial infections. Studies in pneumonia, II - - - - - 293
 KOLMER, JOHN A., COHEN, S., SOLIS-, and STEINFELD, EDWARD. The influence of quinin and urea hydrochlorid, ethylhydrocuprein, and other cinchona derivatives on leukocytosis and on phagocytosis and pneumococci in vitro. Studies in pneumonia, IV - - - - - 333
 KOLMER, JOHN A., SCHAMBERG, JAY F., and RAIZISS, GEORGE D. Various methods of determining the trypanocidal activity of substances in vitro and their relationship to the chemotherapy of experimental trypanosomiasis - - - - - 10
 KOLMER, JOHN A., SCHAMBERG, JAY F., and RAIZISS, GEORGE D. The numeric relationship of infection to the chemotherapy of experimental trypanosomiasis - - - - - 35
 KOLMER, JOHN A. See STEINFELD.

L

- Leukopenia and leukocytosis in rabbits - - - - - 219

M

- MANHEIMER, WALLACE A. Comparison of methods for disinfecting swimming pools - - - - - 1
 Marmots, plague among - - - - - 170
 MATHERS, GEORGE. The etiology of acute epidemic poliomyelitis (Plates 1 to 4) - - - - - 113
 McNAIR, JAMES B. The oxidase of *Rhus diversiloba* - - - - - 485

	PAGE
MEYER, K. F., and CHRISTIANSEN, C. R. The intracutaneous 'typhoidin' reaction.	
I. The preparation and properties of the antigen - - -	357
II. The nature and specificity of the reaction - - -	391
III. The relation of cutaneous hypersensitiveness to experimental immunity and infection - - -	424
Microorganisms, parasitic, adaptation to lowered oxygen tension - -	28

N

NOVY, F. G., DEKRUIF, P. H., and NOVY, R. L. Anaphylatoxin and anaphylaxis. I. Trypanosome anaphylatoxin - - -	499
NOVY, F. G., and DEKRUIF, P. H. Anaphylatoxin and anaphylaxis. II. Agar anaphylatoxin: guinea-pig serum - - -	536
NOVY, F. G., and DEKRUIF, P. H. Anaphylatoxin and anaphylaxis. III. Agar anaphylatoxin: rabbit serum - - -	566
NOVY, F. G., and DEKRUIF, P. H. Anaphylatoxin and anaphylatoxis. IV. Agar anaphylatoxin: rat serum - - -	589
NOVY, F. G., and DEKRUIF, P. H. Anaphylatoxin and anaphylatoxis. V. Effect of multiple doses of anaphylatoxin - - -	618
NOVY, F. G., and DEKRUIF, P. H. Anaphylatoxin and anaphylaxis. VI. Effect of intravenous injections of agar - - -	629
NOVY, F. G., DEKRUIF, P. H., and NOVY, F. O. Anaphylatoxin and anaphylaxis. VII. Peptone anaphylatoxin - - -	657
NOVY, F. G., and DEKRUIF, P. H. Anaphylatoxin and anaphylaxis. IX. Specific anaphylactic shock - - -	776
NOVY, F. O. See NOVY, F. G.	

O

OLITSKY, PETER K., and DENZER, BERNARD S. The passive transference of nonspecific antibodies - - -	145
OLITSKY, PETER K. The nonfiltrability of typhus virus - - -	349
OLITSKY, PETER K. See DENZER.	
OLIVER, WADE W. See WHERRY.	
Oxidase of <i>Rhus diversiloba</i> - - -	485
Oxygen, adaptation of parasitic microorganisms to lower tension of -	28

P

Paratyphoid-enteritidis Group I, differentiation of - - -	457
Pemphigus neonatorum, bacteriology of - - -	86
Peptone, anaphylatoxin - - -	657
Plague pneumonic and septicemic, among marmots - - -	170
Plague poisons and virulence - - -	180
PLOTZ, HARRY. See BAEHR.	
Pneumococci, action of quinin, urea hydrochlorid, ethylhydrocuprein, and other cinchona derivatives on - - -	272, 333
Pneumococci, allergic skin reactions in pneumonia with type strains of -	344
Pneumococcus infections, experimental value of quinin and urea hydrochlorid, ethylhydrocuprein and other cinchona derivatives in - -	313

	PAGE
Pneumonia, skin reaction in - - - - -	344
Pneumonia, studies in - - - - -	272, 293, 313, 333, 344
Poliomyelitis, acute epidemic, etiology of - - - - -	113

Q

Quinin, action on pneumococci in vitro - - - - -	272, 333
Quinin in experimental pneumococcus infection - - - - -	313

R

Rabbits, leukopenia and leukocytosis in - - - - -	219
Rabbit serum, agar anaphylatoxin of - - - - -	566
Rabbits, treatment of experimental tuberculosis in, with taurin, gold chlorid and sodium oleate - - - - -	442
RAIZISS, GEORGE D. - - - - -	10, 35
Rat serum, agar anaphylatoxin of - - - - -	589
Rhus diversiloba, oxidase of - - - - -	485
Russia, blood culture studies on typhus exanthematicus in - - - - -	201

S

SADLER, WILFRID, and KELSO, R. F. An epidemic of acute diarrhea approaching in some cases a mild dysentery - - - - -	140
SCHAMBERG, JAY F. - - - - -	10, 35
Serbia, blood culture studies on typhus exanthematicus in - - - - -	201
Serum, guinea-pig, agar anaphylatoxin of - - - - -	536
Serum, normal, primary toxicity of - - - - -	717
Serum, rabbit, anaphylatoxin of - - - - -	566
Serum, rat, agar anaphylatoxin of - - - - -	589
SHERWOOD, NOBLE P. The effect of various chemical substances on the hemolytic reaction - - - - -	185
Shock, specific anaphylactic - - - - -	776
Skin reactions in pneumonia - - - - -	344
SMILLIE, WILSON GEORGE. Studies of the beta hemolytic streptococcus (Smith and Brown) - - - - -	45
Sodium oleate treatment of experimental tuberculosis combined with taurin - - - - -	442
SOLIS-COHEN, MEYER. See COHEN, MEYER, SOLIS-.	
SOLIS-COHEN, S. See COHEN, S. SOLIS-.	
STEINFELD, EDWARD, and KOLMER, JOHN A. Allergic skin reactions in pneumonia with type strains of pneumococci. Studies in pneumonia. V - - - - -	344
Streptococcus, hemolytic beta, studies of - - - - -	45
Swimming pools, disinfection of - - - - -	1

T

TAKEOKA, MINORICHI. The treatment of experimental tuberculosis in guinea-pigs and rabbits with taurin, alone and in combination with gold chlorid and sodium oleate - - - - -	442
Taurin, Treatment of experimental tuberculosis with - - - - -	442
TEH, WU LIEN. See EBERSON.	

	PAGE
TODD, L. C. Comparative study of <i>Bacillus anthracis-symptomatici</i> and allied organisms with respect to gas production - - - - -	151
Toxicity, primary, of normal serum - - - - -	717
Toxin formation in diphtheria bacillus - - - - -	244
Trypanocidal activity of substances in vitro, methods of determining -	10
Trypanosomiasis, chemotherapy of experimental - - - - -	10
Trypanosomiasis, experimental, numeric relationship of infection to the chemotherapy of - - - - -	35
Tuberculosis, experimental, treatment with taurin, gold chlorid and sodium oleate - - - - -	442
Tuberculin hypersensitiveness as determined by intracutaneous tests of different degree - - - - -	233
Typhoid fever, contact epidemic of - - - - -	109
Typhoidin reaction, intracutaneous - - - - -	357
Typhoidin reaction, nature and specificity of - - - - -	391
Typhus exanthematicus, blood culture studies on - - - - -	201
Typhus exanthematicus, studies on immunity with reference to the anti-bodies in man and guinea-pigs demonstrable by the Dale method -	99
Typhus virus, nonfiltrability of - - - - -	349
Thyroid gland, alleged detoxicating power of - - - - -	131

U

Urea hydrochlorid, action of, on pneumococci in vitro - - -	272, 333
Urea hydrochlorid in experimental pneumococcus infections - - -	313
Virulence of diphtheria bacillus - - - - -	244

W

WEAVER, GEORGE H. The virulence of diphtheria bacilli from diphtheria patients and diphtheria carriers - - - - -	125
WELLS, CLIFFORD W. Leukopenia and leukocytosis in rabbits - - -	219
WHERRY, WILLIAM B., and OLIVER, WADE W. Further observations on the adaptation of parasitic microorganisms to a lowered oxygen tension	28

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